Volume 120 Number 2

THE UNI OF MICHIGAN
MAY 1961
SCIENCE LIBRARY
QH 301 B59
PR

THE BIOLOGICAL BULLETIN

PUBLISHED BY
THE MARINE BIOLOGICAL LABORATORY

Editorial Board

DAVID W. BISHOP, Carnegie Institution of Washington
FRANK A. BROWN, JR., Northwestern University
JAMES CASE, State University of Iowa
JOHN W. GOWEN, Iowa State College
LIBBIE H. HYMAN, American Museum of Natural History
J. LOGAN IRVIN, University of North Carolina
JOHN H. LOCHHEAD, University of Vermont
V. L. LOOSANOFF, U. S. Fish and Wildlife Service
C. L. PROSSER, University of Illinois
BERTA SCHARRER, Albert Einstein College of Medicine
FRANZ SCHRADER, Duke University
WM. RANDOLPH TAYLOR, University of Michigan
DONALD P. COSTELLO, University of North Carolina
Managing Editor

APRIL, 1961

Printed and Issued by
LANCASTER PRESS, Inc.
PRINCE & LEMON STS.
LANCASTER, PA.

INSTRUCTIONS TO AUTHORS

The Biological Bulletin accepts papers on a variety of subjects of biological interest. In general, however, review papers (except those written at the specific invitation of the Editorial Board), very short papers, preliminary notes, and papers which describe only a new technique or method without presenting substantial quantities of data resulting from the use of the new method cannot be accepted for publication. A paper will usually appear within three months of the date of its acceptance.

The Editorial Board requests that manuscripts conform to the requirements set below; those manuscripts which do not conform will be returned to authors for correction before review by the Board.

1. ***Manuscripts.*** Manuscripts must be typed in double spacing (*including* figure legends, foot-notes, bibliography, etc.) on one side of 16- or 20-lb. bond paper, 8½ by 11 inches. They should be carefully proof-read before being submitted and all typographical errors corrected legibly in black ink. Pages should be numbered. A left-hand margin of at least 1½ inches should be allowed.

2. ***Tables, Foot-Notes, Figure Legends, etc.*** Tables should be typed on separate sheets and placed in correct sequence in the text. Because of the high cost of setting such material in type, authors are earnestly requested to limit tabular material as much as possible. Similarly, foot-notes to tables should be avoided wherever possible. If they are essential, they should be indicated by asterisks, daggers, etc., rather than by numbers. Foot-notes in the body of the text should also be avoided unless they are absolutely necessary, and the material incorporated into the text. Text foot-notes should be *numbered* consecutively and typed *double*-spaced on a separate sheet. Explanations of figures should be typed double-spaced and placed on separate sheets at the end of the paper.

3. A ***condensed title*** or running head of no more than 35 letters and spaces should be included.

4. ***Literature Cited.*** The list of papers cited should conform **exactly** to the style set in a recent issue of The Biological Bulletin; this list should be headed LITERATURE CITED, and typed *double*-spaced on separate pages.

5. ***Figures.*** The dimensions of the printed page, 5 by 7⅜ inches, should be kept in mind in preparing figures for publication. Illustrations should be large enough so that all details will be clear after appropriate reduction, but not larger than 15 × 22 inches. Explanatory matter should be included in legends as far as possible, not lettered on the illustrations. Figures should be prepared for reproduction as line cuts or halftones; other methods will be used only at the author's expense. Figures to be reproduced as line cuts should be drawn in black ink on white paper, good quality tracing cloth or blue-lined coordinate paper; those to be reproduced as halftones should be mounted on Bristol Board, and any designating numbers or letters should be made directly on the figures. *All* figures should be numbered in consecutive order, with no distinction between text- and plate-figures. The author's name should appear on the reverse side of all figures, as well as the desired reduction.

6. ***Mailing.*** Manuscripts should be packed flat. **All illustrations larger than 8½ by 11 inches must be accompanied by photographic reproductions or tracings that may be folded to page size.**

Reprints. Reprints may be obtained at cost; approximate prices will be furnished by the Managing Editor upon request.

THE BIOLOGICAL BULLETIN

THE BIOLOGICAL BULLETIN is issued six times a year at the Lancaster Press, Inc., Prince and Lemon Streets, Lancaster, Pennsylvania.

Subscriptions and similar matter should be addressed to The Biological Bulletin, Marine Biological Laboratory, Woods Hole, Massachusetts. Agent for Great Britain: Wheldon and Wesley, Limited, 2, 3 and 4 Arthur Street, New Oxford Street, London, W. C. 2. Single numbers, $2.50. Subscription per volume (three issues), $6.00.

Communications relative to manuscripts should be sent to Dr. Donald P. Costello, Marine Biological Laboratory, Woods Hole, Massachusetts, between June 15 and September 1, and to Dr. Donald P. Costello, P. O. Box 429, Chapel Hill, North Carolina, during the remainder of the year.

Copyright © 1961, by the Marine Biological Laboratory

Second-class postage paid at Lancaster, Pa.

Vol. 120, No. 2 April, 1961

THE
BIOLOGICAL BULLETIN

PUBLISHED BY THE MARINE BIOLOGICAL LABORATORY

OBSERVATIONS ON THE RESPIRATORY ENZYMES OF VARIOUS LIFE-STAGES OF CHIRONOMUS PLUMOSUS, CHIRONOMUS STAEGERI, AND AEDES AEGYPTI[1]

JOHN M. AUGENFELD AND JOHN C. NEESS

Department of Zoology, University of Wisconsin, Madison 6, Wisconsin

The pattern of respiratory enzymes in the midge genus *Chironomus* (*Tendipes*) is of interest for several reasons.[2] Most of the insect tissues investigated by Zebe and McShan (1957) exhibited very little activity of lactic dehydrogenase, an enzyme essential to the operation of anaerobic glycolysis in vertebrate tissue. Yet the larvae of some species of *Chironomus* regularly survive weeks or months in oxygen-free surroundings at the bottoms of eutrophic lakes. Furthermore, the various stages in these insects' life-histories differ markedly in their requirements for energy and in their tolerance of anoxia.

This report describes some measurements of the activity of the anaerobic glycolytic system in larvae of two species of the genus *Chironomus*, and of the succinoxidase and cytochrome oxidase systems in larvae, pupae, and adults of these species and of the mosquito, *Aedes aegypti*.

We are indebted to Dr. William H. McShan for many valuable suggestions, for the use of equipment, for assistance in choosing many of the techniques employed and for training in their use.

Materials and Methods

A. Animal material

The larvae of *C. plumosus* and of *C. staegeri* are found in great abundance burrowing in the bottom sediments of stratified eutrophic lakes where they are frequently subjected to several weeks or more of oxygen deprivation in late summer and fall. In the laboratory, larvae of *C. plumosus* are more resistant to anoxia than those of *C. staegeri* (Neess and Della Croce, unpublished data).

[1] The work described here received financial assistance at various times from the United States Atomic Energy Commission, the National Science Foundation, and the Wisconsin Alumni Research Foundation, whose support is gratefully acknowledged. It is part of the material of a Ph.D. thesis submitted by the senior author to the Department of Zoology, University of Wisconsin.

[2] For a discussion of the use of the name *Chironomus* the reader is referred to Neess and Dugdale (1959).

Copyright © 1961, by the Marine Biological Laboratory

After a period of larval development, lasting up to two years for *C. plumosus* and for an unknown but probably shorter time for *C. staegeri*, the first indications of imminent pupation and emergence appear. At this time, the animals are marked by a darker coloration of the anterior segments and by the appearance of black pigment at the site of the adult eyes. Larvae which show no external changes preparatory to pupation will be referred to hereafter as "normal" larvae, while those in the stage just described will be designated as "pre-pre-pupational." This stage, which may last for several weeks, is followed immediately before pupation by the "pre-pupational," in which the thoracic segments become markedly swollen at the site of the future wing muscles and take on a distinctly lighter color than the rest of the animal. Often groups of pre-pupational larvae exhibit a bimodal weight distribution which Dugdale (1956) has shown to be correlated with sex, the heavier animals representing the future females.

These later larval stages are somewhat more sensitive to oxygen lack than are the earlier stages. They also seem to be more active since they are sometimes taken, apparently while swimming towards the surface, in plankton nets. In both traits, they foreshadow the pupae, which are completely intolerant of oxygen lack and which swim actively towards the surface in preparation for emergence.

The final transformation of *Chironomus* pupae into adults takes place at the surface of the lake, usually at dawn or dusk. The freshly emerged adults rest on the surface of the water briefly and then fly towards shore, where they mate during the day. The males are more active in this process, forming large swarms whose members hover rapidly for extended periods. The females rest on the ground or on leaves most of the time, but occasionally one flies through the swarm, copulates in flight, and returns to rest. At dusk the females fly back onto the lake and lay their eggs in compact sticky masses on its surface. Females which have not laid their eggs are here designated as pre-ovulatory. Those that have laid their eggs are designated as post-ovulatory. The subsequent fate of the females is not known, though the results of this investigation may shed some light on the question. The adults do not live for more than several days after emerging from the lake.

Aedes aegypti also undergoes a transformation from a less active larva through a more active pupa to a highly active adult, but it does not change in its oxygen requirements, being an obligate aerobe at all stages.

Aedes were reared from eggs kindly provided by the U. S. Public Health Service Laboratories of the Communicable Disease Center at Savannah, Georgia. Larvae and pupae of *Chironomus* were obtained by dredging bottom mud from Lake Mendota near Madison, Wisconsin, with a modified Ekman dredge (Welch, 1948, p. 176) and washing it through a wire-mesh screen with a spray of water. Adult *Chironomus* were obtained immediately after their emergence and while the females were laying eggs, by attracting them to a lantern on a boat. Swarming males and females on the ground were captured by netting. All adults were used within six hours and all larvae and pupae within two days after they were collected.

B. Methods

Enzyme activities were generally determined by Warburg manometry, using tissue homogenates. These were prepared by placing weighed live animals into

a sharp-pointed ground glass homogenizer, adding ice-cold distilled water, and grinding with a motor-driven pestle. All tissues were kept in ice after being homogenized. No more than fifteen and usually less than five minutes elapsed between the time the animals were killed and the beginning of the enzyme determination. Readings of pressure changes were made at ten-minute intervals, and the average of the first four or six readings was used. Two to four animals were homogenized together to reduce the effect of individual variations in activity. Larvae and pupae were blotted on filter paper before being weighed to remove the film of water which clings to their surfaces. Adult insects were chilled to prevent them from flying before being weighed and placed in the cup.

The activity of the glycolytic enzyme system was determined by the technique of Novikoff (1948), using a 95% nitrogen, 5% carbon dioxide atmosphere. After the incubation period, the contents of one-half of the Warburg flasks were deproteinized by the addition of equal volumes of 10% perchloric acid and centrifuging, and the amounts of lactic acid formed in these flasks were then determined by the method of Barker and Summerson (1941).

Succinoxidase activities were measured by the method of Schneider and Potter (1943). In some cases, cytochrome *c* or the substrate, sodium succinate, was omitted. Cytochrome oxidase was studied principally by the method of Schneider and Potter (1943), using sodium ascorbate as the substrate. Corrections were made for the autoxidation of the ascorbate by using different amounts of tissue in the individual flasks of a series, and calculating the autoxidation by extrapolating the total oxidation to a tissue concentration of zero. A few determinations of cytochrome oxidase activity were made by the microspectrophotometric method of Cooperstein and Lazarow (1951). All chemicals used were reagent grade commercial preparations.

All the determinations of glycolytic and cytochrome oxidase activities and of the succinoxidase activities of *C. staegeri* and of *A. aegypti* were made at 38° C. Two series of measurements of the succinoxidase activity of *C. plumosus* were made. The first, which included observations on a greater number and variety of adults than the second, was made at 15° C., approximately the highest temperature which the larvae experience in their natural habitat. The second, at which all stages exhibited greater activity, was made at 38° C.

All results are expressed in terms of gas exchange or lactate production per unit wet weight of tissue. Those dry weights which were determined represented a quite constant proportion of the animals' wet weights, ranging from 13% for *C. plumosus* larvae to 26% for *C. plumosus* adults. The final concentrations of tissue in the flasks were between 0.3 and 20.0 mg. wet weight per ml. The concentrations of the different tissues used were chosen so that the changes in pressure would be of a conveniently measured magnitude.

In some cases, the concentration of tissue within the flasks was varied among replicated determinations in order to observe the effects of such variations on the activity. Succinoxidase activity proved to be related to tissue concentration in an almost linear manner in all cases in which the effect was measured. Variations in the tissue concentrations in the determinations of cytochrome oxidase were used in calculating the substrate oxidation rate, and so an assumption of linear response to such variation is necessary. In all cases, variations in unit activity caused by

variations in concentration would contribute to the standard error which was calculated for the mean activity of each tissue. Therefore, it is reasonable to assume that a small standard error or a statistically significant difference between the means of two tissue types indicates that differences in concentration did not affect unit activities significantly.

Results and Discussion

A. Glycolysis

The formation of lactate and the evolution of CO_2 by the larval tissue homogenates in the first series of experiments indicated the presence of a glycolytic system. The omission of both hexose diphosphate and glucose, *i.e.*, of all carbohydrate substrates, reduced the rate of lactate production by 90% while the carbon dioxide production was reduced by only 45%. This indicates that a source of hydrogen ions other than lactic acid was present in the reaction, since any reduction in pH would cause the release of CO_2 from the bicarbonate in the flasks. Since the evolution of CO_2 was prevented only by the omission of ATP from the vessels it is probable that the larvae, like the cockroach and grasshopper muscle studied, respectively, by Barron and Tahmisian (1948) and Humphrey and Siggins (1949) contain an ATP-ase capable of removing an acidic phosphate group from this nucleotide. Therefore, conclusions about the glycolytic capabilities of the larvae have been based on lactate production rather than on the evolution of CO_2.

One indication was found of a qualitative difference between the larval enzyme pattern and those found by Novikoff (1948) in tumors and Utter *et al.* (1945a, 1945b) in mammalian nervous system tissues. In the latter cases the addition of phosphorylated hexose was indispensable for the operation of the system. The rate of production of lactate of three samples of larval tissues, incubated without added HDP, was lowered by only about 40%.

B. Succinoxidase

Table I shows the means and standard errors for oxygen uptake of the various preparations. The endogenous oxidation of homogenates of samples of all the life stages of *C. plumosus* was quite low at 15°, and there were no differences among them. The effect of the omission of cytochrome *c* from the reaction mixtures was less in the less active tissues, *e.g.*, those from larvae and pre-ovulatory females (15°), while in the more active tissues the oxygen uptake was reduced by the same proportion as that found by Potter (1941) and by Schneider and Potter (1943) in rat liver under the same conditions.

At 15°, the succinoxidase activity of homogenates of larvae of *C. plumosus* was little greater than their endogenous oxidation. The pupae showed a highly significant increase in activity over the larvae and a highly significant difference between the sexes, the male pupae having an oxygen uptake over twice that of the female. The rate of oxygen uptake of freshly emerged adult males was over twice that shown by the male pupae, and that of actively swarming males was slightly higher yet. However, the activities of freshly emerged female adults showed no similar increases, but rather fell to levels approaching those of larvae.

Harvey and Beck (1953) and McShan, Kramer and Schlegel (1954) had also found differences in succinoxidase activity between the sexes in an insect, the cockroach *Periplaneta americana*, and Barron and Tahmisian (1948) found a sexual difference in the over-all oxygen uptake of *Periplaneta* thoracic muscle. In these instances the ratio of oxidative activity in the male to that in the female was only two or three, whereas adult male chironomids displayed a rate ten times that of the females. At first it was thought that this difference was correlated with the fact that the difference between the activities of the sexes is greater in *Chironomus* than in *Periplaneta*. This explanation was shown to be incorrect when homog-

TABLE I

Succinoxidase activity in cm.³/gm. wet weight/hr.

Life stage	*C. plumosus*, 15°	*C. plumosus*, 38°	*C. staegeri*, 38°	*A. aegypti*, 38°
Larvae				
"Normal"	.43 ±.05	1.59 ±.06	2.92 ±.10	.72 ±.01 (2 days after hatching)
	.13*	.45**	2.66 ±.12 (sw.)	1.51 ±.22 (5 days after hatching)
	.41 ±.15**			2.35 ±.07 (6–13 days after hatching)
				.68 ±.15 (6–13 days after hatching)*
				.91 ±.51 (6–13 days after hatching)**
Pre-pre-pupational, ♂		1.84 ±.08		
Pre-pre-pupational, ♀		1.89 ±.08		
Pre-pupational, ♂		2.24 ±.10	3.60 ±.04	
		0*		
Pre-pupational, ♀		1.94 ±.10	3.32. ±11	
		0*		
Pupae, ♂	2.54 ±.18	12.26 ±.28	9.22 ±1.15	2.79 ±.09 (♂ + ♀)
	.12*			.91 ±.04* (♂ + ♀)
	1.09**			.65 ±.04** (♂ + ♀)
Pupae, ♀	1.13 ±.04	8.24 ±.34	7.21 ±.28	
Adult, ♂				
Freshly emerged	5.85 ±.32			15.29 ±.61
	.15 ±.02*			
	2.35 ±.33**			
	11.7 ±.23†			
Swarming	7.65 ±.93			
	4.20 ±.37**			
With eggs	.42 ±.03			
Adult, ♀				14.82 ±.17
Freshly emerged	.59 ±.23			12.04 ±.41 (after blood meal)
	.18**			
	6.91 ±.80†			
Pre-ovulatory	.46 ±.05	2.08 ±.66		
	.45 ±.06**	16.70 ±3.3†		
Post-ovulatory				
Lake caught	1.43 ±.40			
Land caught	3.79 ±.26			
	2.43 ±.46**			

* =no substrate; ** =no cytochrome; † =heads and thoraces; (sw.) =swimming.

enates of females which had laid their eggs were found to have a succinoxidase activity some seven times higher than homogenates containing unlaid eggs.

The hypothesis that the eggs of *C. plumosus* were capable of inhibiting the activity of the succinoxidase enzyme system was supported by the fact that homogenates of thoraces, alone, of pre-ovulatory females displayed a succinoxidase activity of the same order of magnitude as homogenates of the entire post-ovulatory animals, which suggested that there was no change in the enzyme system itself at the time the eggs were laid. The inhibitory effect of the eggs was directly demonstrated by homogenizing an egg mass, collected immediately after it had been laid, together with adult male midges in a ratio of two parts by weight of egg mass to five parts of whole male insect. This was approximately the ratio of the weight of a

gravid abdomen to the weight of an adult female. The homogenate of male adult with eggs had an activity as low as that of the entire pre-ovulatory females, while a control without eggs showed a typically high activity. Since the egg masses made up only 40% of the weight of tissue present, while their presence reduced the succinoxidase activity by about 90%, it seems probable that they were actively inhibiting the enzyme system and not merely diluting it with inert tissue.

As Table II indicates, the developed egg masses, when homogenized with rat liver in the same proportion as with male *Chironomus*, reduced its activity by 70%, both at 15° and at 38°, but the egg-containing abdomens of the females, similarly homogenized, had no such effect. This difference may well be related to changes in the chemistry of the egg masses following fertilization or oviposition. The difference between the response to the undeveloped eggs by the tissues of the females and by the rat liver, and the greater magnitude of the effect of the eggs on the adult males may indicate a specific difference in the properties of the enzymes of the two forms. It remains to be seen whether the inhibiting effect of the egg masses has any functional significance for the insects.

TABLE II

Inhibition of mammalian succinoxidase; activity expressed as cm.³ O_2/gm. wet weight/hr.

Tissue and temperature	Control	With *C. plumosus* egg-laden abdomen	With *C. plumosus* egg masses
Rat liver 15°	4.06	4.02 ± .27	1.09 ± .52
Rat liver 38°	25.68 ± 1.34	21.04 ± 1.26	4.89 ± .65
		With *A. aegypti* egg-laden abdomen	With *A. aegypti* eggs
Mouse liver 38°	32.49 ± 1.39	41.05 ± .05	30.34 ± .38

Female midges captured on the shore were readily identifiable as pre-ovulatory or post-ovulatory by the shape of their abdomens, those of the former being swollen and round in cross-section while those of the latter were flat and narrow. The succinoxidase activity of all the members of the former group was distinctively low and that of the latter high. The condition of the midges captured on the lake was more ambiguous. A number of these animals were classified as post-ovulatory whose abdomens were not as completely reduced as those of the animals on shore or which were taken immediately after they were seen to lay eggs. The succinoxidase activity of this group of "post-ovulatory" females was significantly lower than that of the animals taken on land and it showed greater variability. These results seem to indicate that the female midge lays a number of egg masses in separate places on the lake and then returns to shore.

The elimination of the inhibition by the egg masses did not entirely destroy the difference between the sexes, but it did reduce it to the same order as that found in other insects. The rate of oxygen uptake of homogenates of entire swarming males was twice that of the post-ovulatory females. The same ratio was found when the activities of only the heads and thoraces were compared. The absolute

values of the latter were 50–80% higher than those of the entire animals. These results might be expected in view of the very high metabolic rates generally displayed by the thoracic flight muscles of insects. Another indication that the muscles of insects may vary in the level of enzymes of the Krebs cycle was reported by Brooks (1957), who found differences between the succinoxidase activities of different muscles of individuals of the American cockroach as well as between those of the sexes.

At 38°, the activity of the larval succinoxidase was four times that at 15°, indicating a Q_{10} for the system of about two. There was no endogenous oxidation at the higher temperature. Pre-pre-pupational larvae at 38° showed a significantly higher activity than "normal" larvae. There was no difference between the mean activities of the heaviest and lightest individuals of this group, though, as suggested above, they very probably represented female and male. The mean activities of pre-pupational larvae were higher still, and a small, though not significant, difference between the sexes was found. As at 15°, the male pupae showed a sevenfold increase in activity over the larvae, and the female pupae a somewhat smaller increase. The activities of homogenates of whole pre-ovulatory adult females were again little more than those of the larvae, while those of homogenates of thoraces alone were eight times as high.

Tissues from pre-pupational larvae of *C. staegeri*, like those of *C. plumosus*, were somewhat more active than those from "normal" ones, and those from the males were more active than those from the females. The pupae were more active still, but the difference between the activities of pupae and of pre-pupational larvae was less than in *C. plumosus*. The difference in activity between male and female pupae was not significant.

The rate of oxygen uptake by the preparations of *staegeri* larvae was almost twice that shown by preparations of *plumosus* larvae, but the activity of *staegeri* pupae was less than that of *plumosus* pupae. One group of *staegeri* larvae which were taken in a plankton net showed a lower than average rate of succinoxidase activity, perhaps indicating that swimming behavior begins earlier in this form than in *plumosus*.

Since the *Aedes* used in this work were reared in the laboratory from the egg, the development of their enzyme activities could be followed from an earlier stage than that of *Chironomus*. Larvae examined two days after hatching showed a low level of activity, scarcely higher than that of the endogenous oxidation. After five more days of development at a temperature of ca. 20°, the activity had doubled and after eight days, had levelled off at a rate some three times higher than the original one. This final rate was between those shown by larvae of *C. plumosus* and *C. staegeri*.

The omission of cytochrome *c* from homogenates of either larvae or pupae reduced the activity to a level barely above that of the endogenous respiration. The latter was four times higher at a temperature of 38° than the endogenous respiration of *C. plumosus* larvae at 15°.

The pupae of *A. aegypti*, which were not differentiated by sex, had a succinoxidase activity only slightly higher than that of the larvae. The activity of the adults was some six times as high as that of the larvae or pupae and showed no sexual difference.

After a blood meal, the activity per unit weight of the females declined slightly but this may be due to the lower activity of the ingested blood which made up a substantial part of the weight of the insect. Two attempts were made to measure any possible inhibition of succinoxidase by *Aedes* eggs. In the first, mouse liver was homogenized together with dry and presumably viable eggs. In the second, mouse liver was homogenized together with the abdomens of female mosquitoes which had received a blood meal. In neither case was the succinoxidase activity of the mouse liver treated with eggs or abdomens reduced below that of untreated homogenates.

C. Cytochrome oxidase

Table III shows the cytochrome oxidase activity of the various homogenates in cm.^{3} oxygen taken up per gram wet weight per hour. These activities are in all cases greater than the succinoxidase activities of the same tissues, the ratio between

TABLE III

Cytochrome oxidase activity at 38° in cm.^{3}/gm. wet weight/hr.

Life stage	*C. plumosus*	*C. staegeri*	*A. aegypti*
Larvae			
"Normal"			14.97 ± 1.68
10 meters and deeper	7.70 ± .38	21.48 ± .61	
	1.99 ± .35**		
	0*		
9 meters and shallower	13.03 ± .42		
Pre-pupational, ♂	15.89 ± 2.62		
Pre-pupational, ♀	7.13 ± 2.43		
Pupae, ♂	37.90 ± .60	57.07 ± 9.28	25.86 ± .06 (♂ + ♀)
Pupae, ♀	35.75 ± .75	42.16 ± 6.56	
Adult, thorax, ♂	119.70 ± 18.50		

* = no substrate; ** = no cytochrome.

the two varying between three and ten. The changes in activity of cytochrome oxidase during development follow much the same pattern as do those in succinoxidase activity, but there is no evidence for an inhibition of cytochrome oxidase by the developing eggs of *C. plumosus*. The greater individual variations made some of the results less clear-cut.

The "normal" larvae of *C. plumosus* fell into two distinct groups. The majority showed an oxygen uptake of 7.7 ± 0.4 cm.^{3}/g./hr. One group, taken in late September from a relatively shallow area of Lake Mendota in which higher temperatures and oxygen concentrations are found during summer than in the habitat of the first group, had an uptake of 13.0 ± 0.4 cm.^{3}. These larvae showed no external sign of imminent pupation, though animals from this area of the lake generally pupate earlier than those deprived of oxygen earlier in the season. This may indicate either that previous oxygen deprivation reduces the activity of larval cytochrome oxidase or else that changes in cellular respiration preparatory to pupation may precede visible morphological changes.

Male pre-pupational larvae showed a slightly higher cytochrome oxidase activity than the "normal" group from shallow water; females showed a lower, though not significantly lower, activity. As with succinoxidase, the cytochrome oxidase activity of the pupae was some five times higher than that of the larvae and the activity of the male adults was three times that of the male pupae. There was no significant difference between the activities of male and female pupae.

C. staegeri and *A. aegypti* larvae displayed, respectively, three and two times the cytochrome oxidase activity of *C. plumosus* larvae, but the *staegeri* pupae showed only 1.3 times the activity of *plumosus* pupae, and the activity of *Aedes* pupae was less than that of *plumosus* pupae. It is of some interest that both the increases in activity of the two oxidative enzyme systems studied and the reduction of tolerance to anoxia which accompany pupation are greatest in *C. plumosus,* intermediate in *C. staegeri,* and least in *A. aegypti.*

Additional Observations

A small number of observations on the oxidation of reduced cytochrome *c* by homogenates of *C. plumosus* and *C. staegeri* larvae were made using the microspectrophotometric method of Cooperstein and Lazarow (1951). These measurements were made at room temperature, *i.e.*, ca. 25° C. *C. staegeri* was found to have the more active cytochrome oxidase by this method as well, but the difference between the two species was not so great as when measured manometrically.

The effect of cyanide on the total oxidative metabolism of some of the subjects was studied in two ways. The oxygen uptake of intact larvae of *C. staegeri* and *A. aegypti,* maintained without food in a synthetic lake water medium, was measured by Warburg manometry at 20° C. and the effect of concentrations of sodium cyanide varying from 10^{-2} to 10^{-5} *M* was noted. The effect of 10^{-4} and 10^{-5} *M* cyanide on the oxidation of reduced cytochrome *c* by homogenates of larvae of *Chironomus* was also observed.

The addition of 10^{-4} *M* NaCN to their medium reduced the oxygen uptake of live larvae of both species by 30–50%, and higher concentrations caused proportionally greater reductions. Cyanide at 10^{-4} *M* concentration completely blocked the oxidation of cytochrome, and 10^{-5} *M* cyanide reduced it by 70%.

The ability of *C. plumosus* larvae to maintain a normal level of oxygen uptake in the presence of low external concentrations of oxygen, and the extremely low oxygen tensions at which the hemoglobin dissolved in their hemolymph unloads oxygen (at .00079 atmospheres, or 0.6 mm. Hg, according to Fox, 1945) has led to the suggestion (Prosser, 1952, p. 323) that the oxidative enzyme pattern of the larvae may be modified to permit the transfer of oxygen at very low concentrations.

To test this suggestion, two Warburg measurements of cytochrome oxidase were conducted under an atmosphere of 1% oxygen and 99% nitrogen. Under these conditions, no oxygen was taken up, indicating that if an enzyme modification such as that suggested exists, it probably involves a circumvention of cytochrome oxidase rather than an increase in its efficiency or affinity for oxygen. This possibility is also suggested by the fact that some insects are resistant to strong concentrations of cyanide. For example, the strongly aerobic larvae of caddis flies have been observed in this laboratory to survive in 10^{-2} *M* NaCN for more than twenty-four hours. Furthermore, Schneiderman and Williams (1954) have di-

rectly demonstrated a cytochrome oxidase by-pass in the diapausing silkworm pupa. Since much of the respiration of the *Chironomus* larvae studied here was eliminated by treatment with cyanide, it seems that the terminal oxidases of insects display considerable variety.

Summary

1. Larvae of *Chironomus plumosus* and *Chironomus staegeri* have an active glycolytic enzyme system.

2. The succinoxidase and cytochrome oxidase systems of *C. plumosus*, *C. staegeri*, and *Aedes aegypti* are least active in the larval stage, more active in the pupal stage and most active in the adult stage. The increase in activity from larval to pupal stage is greater in those species, *C. plumosus* and *C. staegeri*, in which the larva, but not the pupa, is resistant to anoxia than in *A. aegypti*, in which neither is resistant.

3. Male pupae and adults of *C. plumosus* have a higher rate of succinoxidase activity than females. No sexual difference was found between the cytochrome oxidase activities of pupae or between the succinoxidase activities of *A. aegypti* adults.

4. The developing eggs of *C. plumosus* exert an inhibitory effect on the succinoxidase system of the insect and of rat liver.

Literature Cited

Barker, J. B., and W. H. Summerson, 1941. The colorimetric determination of lactic acid in biological material. *J. Biol. Chem.*, **138**: 535–554.

Barron, E. S. G., and T. N. Tahmisian, 1948. The metabolism of cockroach muscle. *J. Cell. Comp. Physiol.*, **32**: 57–76.

Brooks, M. A., 1957. Succinoxidase in the American cockroach. *Ann. Ent. Soc. Am.*, **50**: 122–125.

Cooperstein, S. J., and A. Lazarow, 1951. A microspectrophotometric method for the determination of cytochrome oxidase. *J. Biol. Chem.*, **189**: 665–670.

Dugdale, R. C., 1956. Studies in the ecology of the benthic Diptera of Lake Mendota. Ph.D. Thesis, University of Wisconsin.

Fox, H. M., 1945. Oxygen affinities of certain invertebrate hemoglobins. *J. Exp. Biol.*, **21**: 161–164.

Harvey, G. T., and S. D. Beck, 1953. Muscle succinoxidase in the American cockroach. *J. Biol. Chem.*, **201**: 765–773.

Humphrey, C. F., and L. Siggins, 1949. Glycolysis in the wing muscle of the grasshopper *Locusta migratoria*. *Australian J. Exp. Biol. Med. Sci.*, **27**: 353–359.

McShan, W. H., S. Kramer and V. Schlegel, 1954. Oxidative enzymes in the thoracic muscles of the woodroach, *Leucophaea maderae*. *Biol. Bull.*, **106**: 341–352.

Neess, J. C., and R. C. Dugdale, 1959. Computations of production for populations of aquatic midges. *Ecology*, **40**: 425–430.

Novikoff, A. B., V. R. Potter and G. A. Le Page, 1948. Glycolysis in tumor homogenates. *Cancer Res.*, **8**: 203–210.

Potter, V. R., 1941. Studies on the mechanism of hydrogen transport in animal tissues. IV. The succinoxidase system. *J. Biol. Chem.*, **141**: 775–787.

Prosser, C. L., D. W. Bishop, F. A. Brown, Jr., T. L. Jahn and V. J. Wulff, 1952. Comparative Animal Physiology. W. B. Saunders Co., Philadelphia.

Schneider, W. C., and V. R. Potter, 1943. The assay of animal tissues for respiratory enzymes. II. Succinic dehydrogenase and cytochrome oxidase. *J. Biol. Chem.*, **149**: 219–227.

SCHNEIDERMAN, H. A. AND C. M. WILLIAMS, 1954. The physiology of insect diapause. VIII. Qualitative changes in the metabolism of the Cecropia silkworm during diapause and development. *Biol. Bull.*, **106**: 210–224.

UTTER, M. F., H. G. WOOD AND J. M. REINER, 1945a. Anaerobic glycolysis in nervous tissue. *J. Biol. Chem.*, **161**: 197–217.

UTTER, M. F., J. M. REINER AND H. G. WOOD, 1945b. Measurements of anaerobic glycolysis in brain as related to poliomyelitis. *J. Exp. Med.*, **82**: 217–226.

WELCH, P. S., 1948. Limnological Methods. Blakiston Co., Philadelphia.

ZEBE, E. C., AND W. H. MCSHAN, 1957. Lactic and α-glycerophosphate dehydrogenase in insects. *J. Gen. Physiol.*, **40**: 779–790.

PHOTOPERIODISM AND THE ANNUAL TESTICULAR CYCLE OF THE BOBOLINK (DOLICHONYX ORYZIVORUS), A TRANS-EQUATORIAL MIGRANT, AS COMPARED WITH TWO TEMPERATE ZONE MIGRANTS[1]

WILLIAM L. ENGELS

Department of Zoology, University of North Carolina, Chapel Hill, N. C.

It is now quite generally accepted that seasonal changes in day-length play an important role in the temporal regulation of several annual cyclic phenomena in many birds of the northern hemisphere. Best known is the effect of photoperiods on the annual cycle of the testes, but evidence is accumulating that there is an effect also on a metabolic cycle apparently peculiar to migratory species, an effect perhaps on migration and perhaps also on the molt cycle (summarized in Farner, 1959).

Transequatorial migrants are of special interest in generalizing from known photoperiodic effects because they are exposed annually to two day-length cycles rather than one (*cf.* Fig. 1), and yet, like all other birds which breed above tropical or subtropical latitudes, they have only one reproductive period each year. Unfortunately, very little experimental work has so far been done on transequatorial migrants. Marshall and Serventy (1959) showed that photoperiods apparently do not influence either the gonadal or molt cycles of a shearwater which, following a breeding season off southeastern Australia, migrates across the equator to the North Pacific Ocean, as far as the Aleutian Islands. Preliminary studies (Engels, 1959) indicated a photoperiodic effect on the testicular cycle of the bobolink (*Dolichonyx oryzivorus*), which breeds in North America above Lat. 40° N. and after a transequatorial migration to South America occupies an area between approximately Lat. 10° and 30° S. These preliminary experiments were in part deficient in design, and certain conclusions drawn from them must be modified in view of the further experimental results now to be reported.

Materials and Methods

Sixteen bobolinks (*Dolichonyx oryzivorus*), 6 slate-colored juncos (*Junco hyemalis*), and 6 white-throated sparrows (*Zonotrichia albicollis*) were used in the experiments. The juncos and white-throated sparrows, representative of Temperate Zone migrants, were captured in February and March from populations wintering in the vicinity of Chapel Hill, North Carolina, and were held through the following summer in an outdoor aviary. Seven of the bobolinks were caught in May on their northward migration, one near Raleigh, N. C., the others near Chapel Hill. Like the juncos and white-throated sparrows, these were held in an outdoor aviary through the summer and thus were exposed to nearly normal

[1] Aided by grants from the National Science Foundation (G-6163) and the Faculty Research Council of the University of North Carolina.

day-lengths until the beginning of experimental lighting on October 1. Nine of the bobolinks were captured in early September near Wilmington, N. C., while on their southward migration, just a few weeks before the start of experimental lighting. Five of the 7 birds taken in May were returned to the aviary in the following

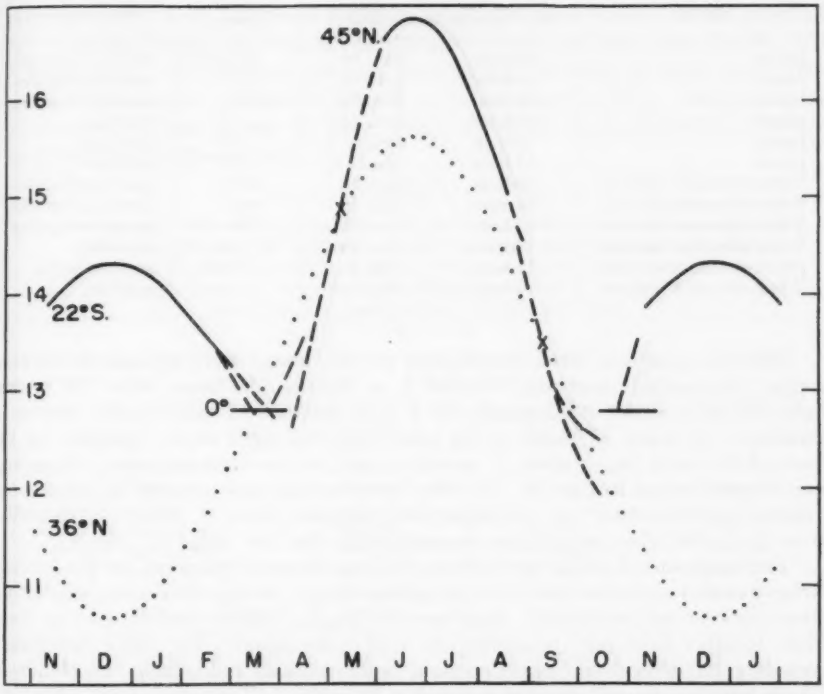


FIGURE 1. Annual cycles of day-lengths (hours between beginning of morning civil twilight and end of evening civil twilight) experienced by bobolinks: *solid lines*, near the center of their "wintering" grounds, at Lat. 22° S.; near the center of their breeding grounds, at Lat. 45° N.; and at the equator, Lat. 0°, during their transequatorial passages; *broken lines*, during migrations (approximate); *x*, time of capture of spring and fall migrants (early May and early September) in North Carolina; *dotted line*, the cycle (at Lat. 36° N.) experienced by captive bobolinks in aviary at Chapel Hill, N. C.—the general form of this cycle is that experienced by all Temperate Zone migrants as well as by non-migrant residents of middle latitudes. Curves for hours between sunrise and sunset would be lower by about one hour, varying from about ¾ hour at the equator and at Lat. 22° S. in December to about 1¼ hours at Lat. 45° N. in June. (From data in: Tables of Sunrise, Sunset and Twilight: Supplement to the American Ephemeris, 1946; U. S. Naval Observatory. Government Printing Office, Washington, D. C.)

May, after 7 to 8 months of artificial day-lengths during which they passed through a complete testicular cycle. They spent the summer again in the outdoor aviary and were used in further experiments beginning in the next autumn—in the tables and discussion these birds are referred to as "second-year experimentals." (Since 5 of the 16 bobolinks were each used twice, there are 21 entries in Table II.)

TABLE I

Responses of juncos and white-throated sparrows to 14-hour photoperiods following 8 (October 1–November 26) of shorter periods; photoperiods from March to October 1 were those natural at Lat. 36° N.

Species	Photoperiod Oct. 1–Nov. 26	Date killed	Vol. of testes	Spermatogenesis
Junco	10-hour	Jan. 16	295 mm.³	sperm in bundles
Junco	10-hour	Jan. 16	247	sperm in bundles
Junco	10-hour	Jan. 16	230	sperm in bundles
Junco	12-hour	Jan. 16	1.2	inactive
Junco	12-hour	Feb. 6	2.1	inactive
Junco	12-hour	March 6	1.2	inactive
White-throated sparrow	10-hour	Jan. 16	440	sperm in bundles
White-throated sparrow	10-hour	Jan. 16	403	sperm in bundles
White-throated sparrow	10-hour	Jan. 16	226	sperm in bundles
White-throated sparrow	12-hour	Jan. 16	2.4	inactive
White-throated sparrow	12-hour	Feb. 6	8.2	spermatocytes
White-throated sparrow	12-hour	March 6	3.1	inactive

Different groups of birds, two to four in each group, were exposed to experimental photoperiods beginning October 1, as follows: 10 hours daily (10 hours light, 14 hours dark) continuously for 7 to 8 months; 14 hours daily, also continuously; 10 hours, 11 hours, or 12 hours daily for eight weeks, followed by 14 hours daily until May (about 6 months); and, in an outdoor aviary, changing day-lengths normal to Lat. 36° N.—this latter regime may properly be termed an experimental treatment for transequatorial migrants since it differs so radically from that which they would have encountered in the free state (*cf.* Fig. 1).

The conditions of confinement during the experimental treatment for the juncos, white-throated sparrows and those bobolinks caught during May were similar to those used in the preliminary experiments (Engels, 1959); that is, two to four birds together in a cage measuring 24 × 24 × 36 inches. The other bobolinks, including the second-year experimentals, were confined individually in Hendryx "finch breeding cages" measuring 8 × 9 × 16 inches. Terramycin was added to the drinking water supplied to these birds. Food, during most of the experiments, consisted of a chick laying mash (Purina Layena); during the last few months we changed to a mash prepared for "game" birds (also Purina).

The development of black pigment in the beak was taken as the criterion of testicular recrudescence in the bobolinks. The first appearance of this pigmentation probably coincides with that stage of the spermatogenic cycle when most of the spermatocytes are undergoing the first meiotic division. However, it should be noted that the observations have to do with evidence of the cycle of production of male sex hormone, which apparently directly causes beak pigmentation (Engels, 1959), and only by inference with the spermatogenic cycle.

EXPERIMENTAL RESULTS

The results of the experiments as given in Tables I and II may be summarized as follows:

Juncos and white-throated sparrows

Valid comparison may be made between these Temperate Zone migrants (Table I) and the bobolinks caught in May and subjected to similar artificial photoperiods (Table II, spring captures, Groups B and D). Exposed to 14-hour photoperiods after 8 weeks of 10-hour photoperiods, juncos and white-throated sparrows had already completed spermatogenesis by January 16, whereas bobolinks first developed beak pigmentation between mid-February and early March, 4 to 7 weeks later. On the same 14-hour photoperiod following 8 weeks of 12-hour photoperiods, testes of juncos and most white-throated sparrows remained in the winter inactive state at least to early March, by which time bobolinks had already developed beak pigmentation.

TABLE II

Response of male bobolinks to various photoperiods; beak pigmentation indicates production of male sex hormone and, inferentially, progress of spermatogenesis at least to first (meiotic) division of spermatocytes. ("Spring captures" and "2nd-year experimentals" experienced the natural day-lengths of Lat. 36° N. for at least four months before start of experiments on October 1)

Group	Category	Photoperiods		Beginning of beak pigment*
		Oct. 1–Nov. 26	Dec.–May	
A	autumn cap.	10-hour	10-hour	May 16
	2nd-yr. exp.	10-hour	10-hour	May 11
B	spring cap.	10-hour	14-hour	Feb. 13
	spring cap.	10-hour	14-hour	Feb. 20
	autumn cap.	10-hour	14-hour	March 12
	autumn cap.	10-hour	14-hour	March 16
	2nd-yr. exp.	10-hour	14-hour	Feb. 17
C	spring cap.	11-hour	14-hour	Feb. 27
	spring cap.	11-hour	14-hour	Feb. 27
D	spring cap.	12-hour	14-hour	Feb. 20
	spring cap.	12-hour	14-hour	March 6
	autumn cap.	12-hour	14-hour	March 2
	autumn cap.	12-hour	14-hour	March 16
	2nd-yr. exp.	12-hour	14-hour	Feb. 17
E	autumn cap.	14-hour	14-hour	—**
	autumn cap.	14-hour	14-hour	—**
	2nd-yr. exp.	14-hour	14-hour	Feb. 17
F	autumn cap.	Natural to Lat. 36° N.		April 20
	autumn cap.	Natural to Lat. 36° N.		April 20
	spring cap.	Natural to Lat. 36° N.		April 1
	2nd-yr. exp.	Natural to Lat. 36° N.		April 1

* Dates indicate end of interval (4 to 7 days) during which beak pigmentation appeared.
** Beak still neutral, May 25.

Bobolinks

Lumping together the three categories in Table II (spring captures, autumn captures, second-year experimentals) for the various groups, we may note the following significant points. When exposed to constant 10-hour photoperiods (Group A), beak pigmentation did not appear until mid-May. When, after 8 weeks on 10-hour photoperiods, the length of the photoperiod was increased to 14 hours, beak pigmentation developed in the various individuals between mid-February and mid-March (Group B), two to three months earlier than in the first group. Similarly, when the 14-hour photoperiod was preceded by 8 weeks of either 11-hour or 12-hour photoperiods, beak pigmentation appeared variously from mid-February to mid-March (Groups C, D). However, on continuous exposure to 14-hour photoperiods from October 1 (Group E), without prolonged previous exposure to short days, only one of three birds (a second-year experimental) developed pigment in the beak; two birds caught a few weeks before treatment began still lacked beak pigmentation in late May, when the experiment was terminated. Bobolinks held in the outdoor aviary (Group F) developed beak pigmentation in April, definitely later than most of the birds on artificial photoperiods, but from 4 to 7 weeks earlier than those continuously on 10-hour photoperiods. Song was heard from the aviary beginning in the latter part of March, when several inches of snow lay on the ground.

In general, beak pigmentation began somewhat later in birds caught in the autumn than in the other categories.

Discussion

Some preliminary experiments on bobolinks (Engels, 1959) seemed to show that the rate of response to 14-hour photoperiods depended inversely on the length of the photoperiod to which males had been exposed previously during the postnuptial, photorefractory phase of their cycle. That is, on 14-hour photoperiods beak pigmentation developed earlier in males which had been previously exposed to 10-hour photoperiods, several weeks later in males previously on 12-hour photoperiods. The difference is not apparent in the present experiments. However, birds used in the earlier study had been held on continuously long photoperiods (16-hour) throughout the summer, rather than on natural day-lengths as in the present study, and it is suggested that this difference in preliminary treatment is responsible for the difference in results. The idea is held, tentatively, that the reinforcement of photorefractoriness induced by some months of previously long days was overcome sooner on the shorter (10-hour) photoperiod. Further study is necessary to determine if we have to do here with any facet of the photoperiodic mechanism as operative in nature.

The relatively rapid response of juncos and white-throated sparrows to long photoperiods in early winter following a few weeks of short photoperiods in autumn (Table I) is characteristic of Temperate Zone migrants. Bobolinks treated in identical manner, however, were quite slow to show a response (Table II, B–D). It would appear that the cycle in these transequatorial migrants strongly resists the accelerating influence of long days, and that some process in the mechanism of response even on long days requires a rather long time, several weeks

longer than in Temperate Zone migrants. Farner (1954) expected such a retardation of testicular recrudescence in response to long days on the part of transequatorial migrants, and he postulated for them (p. 29) "a characteristically long refractory period." Although we may get involved in semantic difficulties here, the present experiments suggest rather that the retardation could be due, at least to some considerable extent, to a very slow rate of response following the termination of refractoriness. In other words, the value of Farner and Wilson's (1957) constant k for testicular growth may be very much lower in bobolinks, especially on long photoperiods, than in Temperature Zone migrants.

Wolfson and Westerhoff (1960) in a short summary report on photoperiodic responses of *Dolichonyx* conclude, as had I previously (Engels, 1959), that a preparatory phase (= photorefractory phase) exists in this transequatorial migrant and that, moreover, this phase "may be regulated by the short days during the period of the autumnal equinox." They further conclude that "compared with temperate species, the duration of the period of short days required may be longer." This seems to me to be an unnecessary extrapolation and quite probably an inaccurate estimate of the operation of the mechanism in nature. In all Temperate Zone migrants so far investigated, the refractory period is terminated in nature by mid-November (Farner, 1954, p. 30; Farner, 1959, p. 732). Study of Figure 1 will show that it would be virtually impossible for migrating bobolinks to continue to experience beyond mid-November the short days of low or middle latitudes north of the equator. (See also final paragraph of this discussion.)

It may be of interest to note especially the responses of those bobolinks designated in Table II as second-year experimentals. These are the same birds which a year earlier were the "spring captures" in Groups B, C and D; as such, following several months of experimental lighting, they all had gone through a relatively sudden post-nuptial molt in May, with accompanying disappearance of the black pigmentation characteristic of the beak in nuptial dress. In nature these events would not have occurred until about two months later. After another summer, then, in the outdoor aviary, they were again exposed to artificial photoperiods as "second-year experimentals." The three which had been distributed one each in Groups B, D and E developed beak pigmentation almost simultaneously during the 7-day interval ending February 17. I would not emphasize the relatively early date nor the coincidence of response (both of which may have some significance), but rather the appearance of beak pigment (also full song) in the one second-year experimental of Group E. This was the only one of 7 male bobolinks (including four birds of the preliminary experiments—Groups A, G; pp. 761, 762, Engels, 1959) on continuously long photoperiods which developed beak pigmentation. We may at least suspect that the abnormally long interval since the last testicular regression, perhaps together with the relatively shorter days of late summer and early fall (see also last paragraph, below), permitted this bird to pass through the refractory phase and eventually to respond to the stimulus of the 14-hour photoperiod.

Despite the obvious difficulty of interpretation of these second-year experimentals, it seems to me that the general picture revealed in these experiments is one which fits the requirements of a photoperiodic mechanism as the timer for annual recrudescence of the testes in a transequatorial migrant: (1) the initiation

of those processes leading to release from photorefractoriness, during the relatively short days of early northern autumn and during the southward passage through the equatorial region; (2) following eventual release from photorefractoriness, regrowth of the testes at a slow rate during the southern hemisphere summer when day-lengths, though declining, are still relatively long; correlated (3) with processes, the regulation of which is largely unknown, leading to migration. Migration itself exposes the birds, once past the equator, to photoperiods of rapidly increasing length (*cf.* Fig. 1).

We may note here that bobolinks held captive in the northern hemisphere (Lat. 36° N.) and exposed to natural day-lengths (and temperatures) showed a revival of the testicular cycle by April (Table II, F). The first flocks of bobolinks (all males) normally arrive from the south at this latitude about May 1, at about the time at which our locally wintering juncos and white-throated sparrows begin their northward migration. Thus, the testicular cycles of these captive bobolinks were approximately in phase, and it may be assumed that if the birds had been released, they could have reached the breeding ground at about the proper time and in breeding condition. It is therefore suggested that, as far as the photoperiodic mechanism is concerned, bobolinks are not required to "winter" below the equator; they could establish a wintering population in the northern hemisphere. The reverse would not seem to be true of such Temperate Zone migrants as juncos and white-throated sparrows, without readjustment of their photoperiodic mechanisms. Both by reason of their inability to overcome refractoriness on intermediate day-lengths (about 12 hours; *cf.* Table I) and their rapid response to long days when released from refractoriness, they probably could not extend their wintering range to and beyond the equator and still maintain that regularity of the annual cycle which leads to the coincidence of one annual reproductive period with a favorable climatic season.

Finally, it should be pointed out that bobolinks may usually not experience such a long period of such relatively short days as used in the present experiments, especially if any appreciable part of the morning and evening twilight periods adds to the effective length of the natural photoperiod (Fig. 1), as is almost certainly true. If so, they must be able to pass through the photorefractory phase and become photoreceptive on photoperiods even longer than 12 hours. It will be of interest to test this possibility.

Summary

1. Bobolinks exposed to 10-hour photoperiods daily through the fall, winter and spring did not develop the beak pigmentation characteristic of the male nuptial dress until early in May. Others exposed at the same time to 14-hour photoperiods daily failed to develop beak pigmentation. But when exposure to 14-hour photoperiods was preceded by 8 weeks (October–November) of 10-, 11- or 12-hour photoperiods daily, pigmentation of the beak began variously between mid-February and mid-March.

2. Juncos and white-throated sparrows exposed to 14-hour daily photoperiods following 8 weeks (October–November) of 10-hour daily photoperiods had completed spermatogenesis by mid-January; but in others for which the preliminary photoperiod had been 12 hours the testes remained inactive.

3. Bobolinks held throughout the fall, winter and spring in an outdoor aviary, on changing day-lengths natural at Lat. 36° N., developed beak pigmentation in April, thus being approximately in phase, in their testicular cycles, with locally wintering juncos and white-throated sparrows.

4. The results are interpreted to mean that bobolinks, unlike typical Temperate Zone migrants, are able to overcome post-nuptial photorefractoriness on days of intermediate length, such as would be encountered during transequatorial migration, but that regrowth of the testes through the long days of the southern hemisphere summer proceeds at a rate much slower than that demonstrated by Temperate Zone migrants on similarly long days, and that full recrudescence is thus delayed, probably beyond the onset of northward migration.

5. It is further suggested that as far as the photoperiodic mechanism is concerned bobolinks could establish a wintering population in the northern hemisphere; but juncos and white-throated sparrows could not extend their wintering range into the southern hemisphere without readjustment of this mechanism.

LITERATURE CITED

ENGELS, W. L., 1959. The influence of different daylengths on the testes of a transequatorial migrant, the Bobolink (*Dolichonyx oryzivorus*). *In:* Photoperiodism and Related Phenomena in Plants and Animals (pp. 759–766). Publ. No. 55, Amer. Assoc. Adv. Sci., Washington, D. C.

FARNER, D. S., 1954. Northward transequatorial migration of birds. *Sci. Rev.* (*New Zealand*), **12**: 29–30.

FARNER, D. S., 1959. Photoperiodic control of annual gonadal cycles in birds. *In:* Photoperiodism and Related Phenomena in Plants and Animals (pp. 717–750). Publ. No. 55, Amer. Assoc. Adv. Sci., Washington, D. C.

FARNER, D. S., AND A. C. WILSON, 1957. A quantitative examination of testicular growth in the white-crowned sparrow. *Biol. Bull.*, **113**: 254–267.

MARSHALL, A. J., AND D. L. SERVENTY, 1959. Experimental demonstration of an internal rhythm of reproduction in a transequatorial migrant (the short-tailed shearwater *Puffinus tenuirostris*). *Nature*, **184**: 1704–1705.

WOLFSON, A., AND T. R. WESTERHOFF, 1960. Photoperiodic regulation of the preparatory phase of the annual gonadal cycle in a transequatorial migrant, *Dolichonyx oryzivorus*. *Anat. Rec.*, **137**: 402.

LUNAR ORIENTATION OF ORCHESTOIDEA CORNICULATA STOUT (AMPHIPODA)

J. T. ENRIGHT

Scripps Institution of Oceanography, La Jolla, California

The celestial navigation accomplished by Talitridae (Amphipoda) was first reported by Pardi and Papi (1953) and Papi and Pardi (1953). These two papers contain numerous observations of the solar navigation of *Talitrus saltator* and, in the second paper, certain night-time observations suggested the existence of lunar navigation in this species, *i.e.*, appropriately time-shifted angles of orientation with the moon. The existence of solar navigation has been confirmed with several other species of Talitridae: *Talorchestia megalophthalma* and *Talorchestia longicornis* (Menaker, 1958); *Talorchestia deshayesei* (Pardi and Grassi, 1955); *Orchestoidea corniculata* and *Orchestoidea benedicti* (unpublished qualitative observations of the author). The existence of lunar navigation, however, had not been completely documented until the recent paper by Papi and Pardi (1959) which presents results supporting the contention that, under the experimental conditions described, *Talitrus saltator* oriented to moonlight with an angle that varied with lunar position. This resulted in a relatively constant and ecologically "correct" compass orientation. On the basis of this evidence, the authors conclude (p. 596) "dass zwei verschiedene physiologische Rhythmen die Sonnen- und die Mondorientierung von *Talitrus* bedingen." (Two different physiological rhythms are responsible for the solar and lunar orientation of *Talitrus*.)

Because of the significance of such a conclusion to the general theory of endogenous rhythms, the present study was undertaken to determine whether *Orchestoidea corniculata* Stout, a related amphipod which is capable of solar navigation, is similarly able to navigate by moonlight.

This work was performed during the tenure of a National Science Foundation predoctoral fellowship, and supported by N.S.F. grant G-7141 to Dr. E. W. Fager. The author wishes to express sincere thanks to his scientific advisor, Dr. E. W. Fager, for encouraging this study and greatly assisting with the manuscript. Appreciation is also due to: Mrs. D. L. Minis (Princeton University) for help with translation; to Mr. A. O. Flechsig, who aided with photography; and to Dr. Leo D. Berner, who demonstrated the calculation of lunar azimuths.

Methods and Materials

The experimental animals were adult and sub-adult specimens of *Orchestoidea corniculata* Stout, collected usually on the morning before the experiment. In a few cases, as indicated in Table I, the animals were collected one or two weeks before the experiment and kept at constant temperature until the day of the

experiment. Following collection, all animals were kept in light-tight, one-liter bottles containing moist sand. The animals can survive two weeks in such containers with no evidence of oxygen shortage. No control of the temperature was attempted on the day of the experiment, but the range was not greater than 16° to 28° C. Collections were made on the beach in front of the Scripps Institution of Oceanography, La Jolla, California. The shoreline there is oriented north 15° east; the "correct" azimuth orientation to return the animals to the water is thus 285° from north.

In order to avoid fog, city sky-glow, surf sounds, etc., the experiments were conducted in a level, open field about 40 km. northeast (inland) of San Diego.

TABLE I

Orientation of Orchestoidea corniculata with moon: animals kept in constant darkness

Date	Time	Lunar stage: days	Lunar azimuth	Orientation azimuth	Vector length	N	Angle with moon
1960							
*9 July	2241	16	137°	258°	0.74	26	121°
9 July	2306	16	141°	266°	0.77	32	125°
**9 July	2330	16	148°	277°	0.40	28	129°
***10 July	0055	16	171°	260°	0.62	29	89°
*10 July	0133	16	183°	280°	0.64	30	97°
10 July	0153	16	189°	307°	0.62	25	118°
*10 July	0330	16	216°	001°	0.64	26	145°
10 July	0355	16	222°	349°	0.65	29	127°
16 July	0144	22	093°	212°	0.59	30	119°
30 July	2003	8	228°	352°	0.53	31	124°
30 July	2055	8	239°	348°	0.56	25	109°
30 July	2105	8	241°	359°	0.62	23	118°
30 July	2147	8	248°	010°	0.58	26	122°
30 July	2158	8	250°	041°	0.65	13	151°
6 August	2201	15	144°	314°	0.72	24	170°
7 August	0005	15	181°	299°	0.85	25	118°
7 August	0017	15	185°	293°	0.61	26	108°
7 August	0240	15	224°	329°	0.65	25	105°
7 August	0252	15	226°	338°	0.79	29	112°

* Animals kept for one week at 20° C.
** Animals kept for one week at 10° C.
*** Animals kept for two weeks at 10° C.

The observation chamber was an opaque, white, circular plastic tray 25 cm. in diameter, 4 cm. deep, over which an unmarked sheet of glass was placed. No attempt was made to control the humidity within the chamber; Papi and Pardi (1959, p. 585) state that this is usually unimportant.[1]

The camera was mounted 1.4 meters above the chamber. Photographic lighting involved a single electronic photo-flash bulb ("strobe light") fixed 1.2 meters above the observation chamber, and at a constant position relative to it. The results suggest that the photographic equipment did not affect orientation. As later discussed, the flashes necessary for picture-taking did have an effect.

[1] See, however, Papi and Pardi (1953, p. 501, 502).

A compass and a clock, out of view of the animals, were included in each photograph.

Results are summarized by the method described by Pardi and Papi (1953, p. 463) : a vector sum of the positions of the individual animals is calculated (using 16 equal sectors), and this is divided by the number of animals. The orientation of the resultant vector gives the average orientation direction and its length gives an indication of the degree of scatter about the average. Such a vector can range in length from zero, indicating an even distribution of directions around the center, to unity, indicating all animals within the same sector. Random distributions will, of course, result in vectors larger than zero.

In order to determine the vector length which would result from random, independent orientation of the animals, 20 "experiments" were performed by assigning positions to 30 hypothetical "animals" by use of a table of random

TABLE II

Orientation of Orchestoidea corniculata *with moon: animals not kept in constant darkness*

Date	Time	Lunar azimuth	Orientation azimuth	Vector length	N	Angle with moon
1960						
6 August	2015	122°	288°	0.59	25	166°
6 August	2025	124°	291°	0.87	21	167°
6 August	2225	151°	339°	0.77	25	188°
6 August	2235	153°	299°	0.72	25	146°
*6 August	2245	156°	319°	0.87	25	163°
*6 August	2255	159°	325°	0.86	30	166°
7 August	0025	187°	321°	0.68	22	134°
7 August	0045	193°	286°	0.47	31	93°
*7 August	0100	198°	325°	0.77	25	127°
*7 August	0110	201°	321°	0.75	26	120°
7 August	0305	229°	280°	0.44	27	51°
7 August	0315	231°	298°	0.64	28	67°
*7 August	0325	233°	341°	0.64	26	108°
*7 August	0335	235°	320°	0.52	25	85°

* "Redarkened" animals; all others "Natural Light."

numbers. Eighty per cent of the resultant vectors were less than 0.30 in length and none was greater than 0.37. This suggests that a vector of length 0.35 or less based on the positions of 20 to 35 animals, the number usually used in the actual experiments, is of doubtful meaning. As there is clearly a lack of independence in the behavior of the individual animals, which predominantly cluster around the edge of the container, and often crowd and crawl over one another, a vector of greater length, perhaps 0.50 or more, is required to indicate definite orientation. No observations were used in the tests of the lunar navigation hypothesis (Tables I and II) if the calculated vector was less than 0.40 in length. The interaction invalidates conventional statistical techniques for more rigorously evaluating variability on the basis of an individual experimental result.

All angles are measured clockwise; azimuths are measured clockwise from north.

Results

Moonlight as the orienting stimulus

In order to determine whether the observed orientations were due to moonlight, a group of amphipods, which had shown a given orientation relative to the moon, was shaded and an image of the moon was presented at about 180° from the actual position of the moon by means of a mirror. The results, shown in Figure 1, clearly demonstrate that the apparent lunar position is the dominant orienting stimulus for *Orchestoidea corniculata* under these experimental conditions.

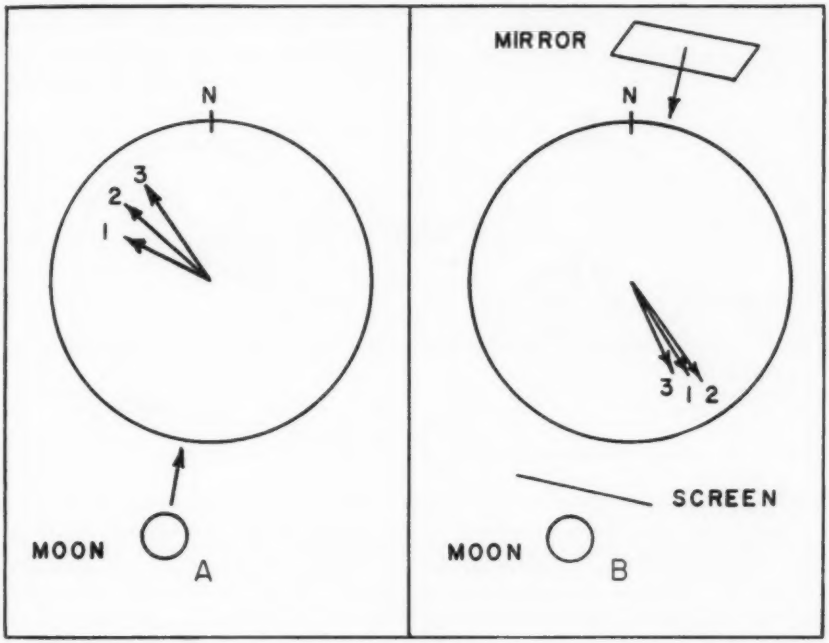


FIGURE 1. Orientation of a group of *Orchestoidea corniculata* to apparent lunar position. A: Initial orientation with the moon; B: orientation with moon reflected at about 180° from true position. Arrows within circle (which has unit radius) indicate average direction and scatter of orientation. Numbers adjacent to arrows indicate sequence in which pictures were taken. Observations made 10 July 1960, 0153–0209 hours, with 25 amphipods.

Serial observations with electronic flash

In initial experiments, a series of three photographs of each group of animals was taken. It was noted that groups of amphipods frequently showed a consistent shift in average direction of orientation, presumably as a reaction to the electronic flash. These results, while too few to be statistically significant, indicated a trend for the animals to orient with successively larger angles with the moon. (See, for example, Fig. 1A.) There was significant evidence of an increase in scatter

from Photograph 1 to Photograph 3. For example, in 19 sets of 3 photographs, Photograph 1 showed least scatter in 14 cases and Photograph 3 showed most scatter in 12 cases.

Two series of ten photographs were taken to determine whether the shifts in direction and increase in scatter are cumulative (Fig. 2). In both cases, the ultimate angle of orientation with the moon after ten photographs was much smaller than the initial angle (contrary to the initial trend, over sets of only three photographs), with a net shift of 85° to 90°. In neither case is the final orientation more "correct" than the initial (although an average of the vectors of Figure 2B would be much closer to "correct" than either initial or final results). The short duration of the experiments, less than 15 minutes in each case, precludes the pos-

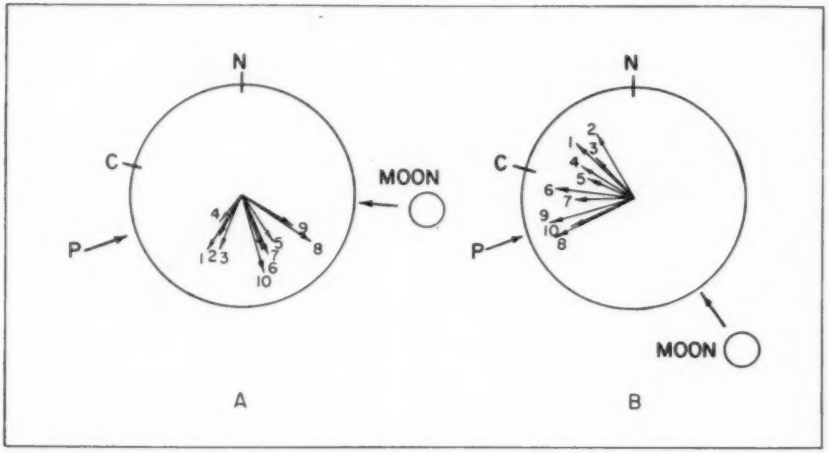


FIGURE 2. Orientation in sequential photographs of two groups of amphipods. A: 16 July 1960, 0144–0158 hours, with 30 amphipods; B: 6 August 1960, 2201–2214 hours, with 24 amphipods. Arrows within circle (unit radius) represent average direction and scatter of orientation; numbers associated with arrows indicate sequence. "P" indicates direction of photoflash; "C" indicates "correct" orientation direction relative to "N," north.

sibility that the shift is due to some normal compensation for changes in lunar position. Furthermore, the shift apparently does not take place without photography: qualitative continuous observation of a group of unphotographed amphipods, kept in the observation chamber for 15 minutes, indicated no noticeable orientation shift. (2 September 1960, 2315–2330 hours; lunar day 12; lunar azimuth 206°–210°; estimated orientation azimuth, initial and final, about 340°; angle with the moon, about 130°.)

The results in Figure 2 seem best interpreted as a resetting effect of light on a rhythmic system, a phenomenon previously demonstrated using similar millisecond electronic flashes[2] (Pittendrigh, in press). The lengths of vectors in

[2] See Pardi and Papi (1953, pp. 466–467) for an alternative explanation of a superficially similar result with solar orientation.

Figure 2 indicate that, after an initial increase in scatter over the first three to five photographs, there is a recovery so that final orientation, after ten photoflashes, is strongly directed, but in a different direction from the initial.

Because of these complications with multiple photographic observations of the moonlight orientation of *Orchestoidea corniculata*, all data subsequently cited consist only of results from first photographs of the initial, unshifted orientation, and no group of amphipods was used twice.

Orientation of amphipods kept in constant darkness

Observations of the orientation of *Orchestoidea corniculata*, kept in complete darkness until just prior to observation, are summarized in Table I and Figure 3. As Figure 3 shows, the data can be explained by the hypothesis that this amphipod, when exposed after ten or more hours of constant darkness, orients to the moon

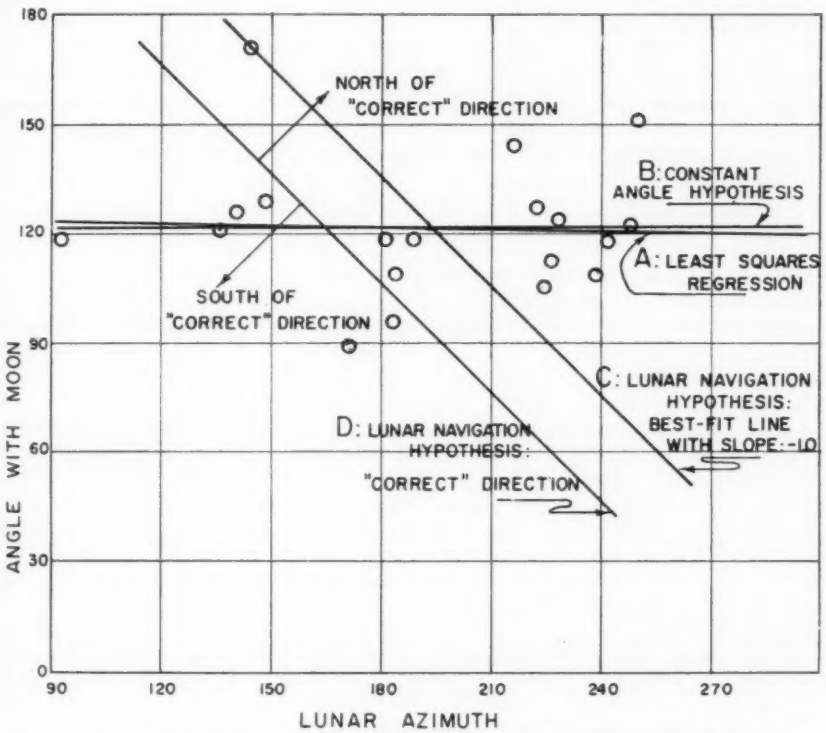


FIGURE 3. Orientation of *Orchestoidea corniculata* with moon, compared with three hypotheses: animals kept in constant darkness. The slope of the least-squares regression line (A) is −0.015. This is not significantly different from zero (line B); ($p > .50$). The probability that the slope of the regression line is as large as −0.50 is less than .001; steeper slopes (−1.0 for line C) are even less likely; and the departure from line D is even greater.

with an average angle of about 120°, regardless of lunar stage or position (Lines A and B). The hypothesis of a *continuously-operating* endogenous lunar physiological rhythm in this species, similar to that claimed for *Talitrus saltator*, appears unnecessary and is contradicted by the data (Lines C and D).

Orientation of amphipods exposed to sunset and moonrise

Additional observations were made with amphipods placed in constant darkness at the time of collection, and then re-exposed, in the sand-containing bottles, to

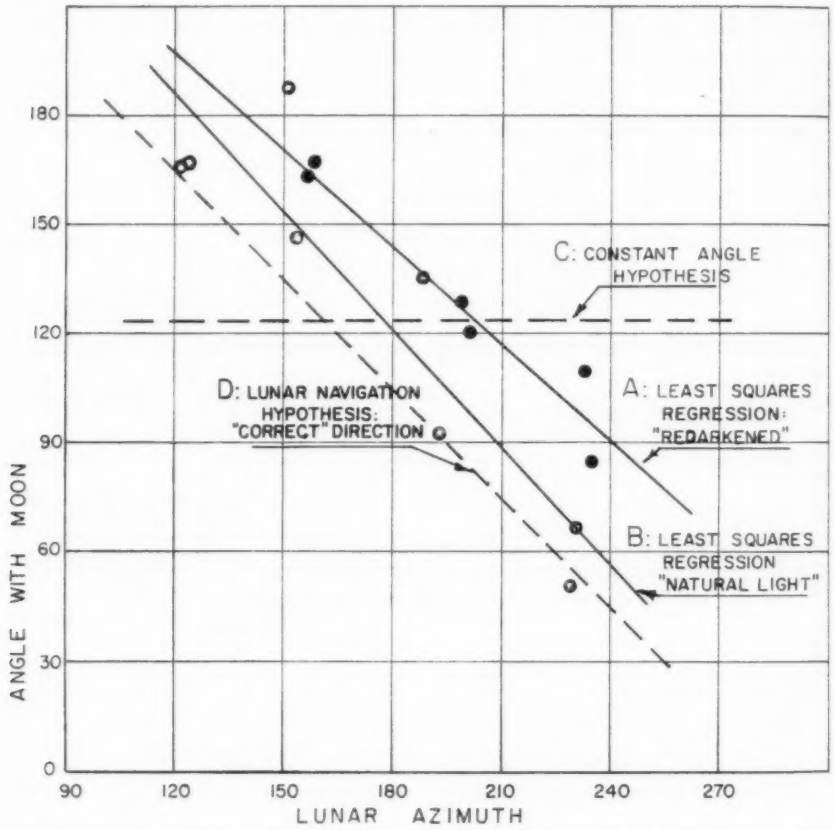


FIGURE 4. Orientation of *Orchestoidea corniculata* with moon as a function of lunar azimuth: animals not kept in constant darkness. Open circles represent orientation of "Natural Light" animals; solid circles represent orientation of "Redarkened" animals. The slope of line A is −0.888; and of line B is −1.078. The probability of the slope being −1.00 is > .20 for line A, and > .50 for line B. Points to the right of line D are north of "correct" orientation.

natural light about one hour prior to sunset. Those which were then continually exposed until observation are designated "Natural Light"; others which were replaced in constant darkness two hours after moonrise are designated "Redarkened." The observations, made August 6 and 7 (full moon), are summarized in Table II and Figure 4. The orientation resulting from this treatment of the animals does not seem adequately explained by the constant-angle hypothesis (Line C); the probability that the slope of the regression is 0 is extremely small in both cases ($p < .001$).

The directions of orientation, particularly those of the "Redarkened" animals, are generally somewhat north of the "correct" 285° azimuth, but the slopes of both regression lines are not significantly different from -1.0, the expected value if the animals compensated appropriately for shifts in lunar position.

A possible explanation is that sunset and/or moonrise (or perhaps removal from constant dark) can initiate a single cycle of appropriately time-compensated lunar orientation in these organisms. Such a system, with no information carry-over from the preceding nights, would afford the animals a crude but workable orientation mechanism (except at first quarter?). It would not require the organizational complexity and long-term precision necessary for the *continuously-operating* endogenous lunar periodicity claimed for *Talitrus saltator*. Further investigation may indicate that "night-sun" orientation (Pardi, 1953/54) or some other by-product of solar navigation is involved. No investigation of the effect of this treatment on the solar navigation of the amphipods was made.

Summary

1. The orientation of *Orchestoidea corniculata* in direct moonlight, compared with the orientation when moonlight is reflected from a mirror, demonstrates that apparent lunar position is the dominant night-time orienting stimulus for this organism.

2. An analysis of the effects of repeated photographic recording of the orientation indicates that this species changes its angle of lunar orientation and the scatter about the average as a result of repeated electronic photo-bulb flashes.

3. Using only single observations of each group of animals, in order to avoid these artifacts, it was determined that this species, kept in total darkness for ten or more hours before observation, orients at a relatively constant angle with the moon, regardless of lunar stage or position. This result is not compatible with the hypothesis that *O. corniculata* possesses a *continuously-operating* lunar physiological rhythm similar to that claimed for *Talitrus saltator*.

4. When the amphipods were exposed to sunset and moonrise on the night of observation, there was an indication of subsequently time-shifted angles of orientation with the moon. The tentative hypothesis of a single-cycle night-time orientation rhythm, re-initiated by the appropriate stimuli each night, would explain such observations.

Literature Cited

Menaker, M., 1958. Celestial time compensated orientation of East Coast amphipods. *Anat. Rec.*, **132**: 476.

Papi, F., and L. Pardi, 1953. Ricerche sull'orientamento di *Talitrus saltator* (Montagu) (Crustacea-Amphipoda). II. *Zeitschr. vergl. Physiol.*, **35**: 490–518.

PAPI, F., AND L. PARDI, 1959. Nuovi Reperti sull'orientamento lunare di *Talitrus saltator* Montagu (Crustacea-Amphipoda). *Zeitschr. vergl. Physiol.*, **41**: 583–596.

PARDI, L., 1953–54. Esperienze sull'orientamento di *Talitrus saltator* (Montagu) (Crustacea-Amphipoda): l'orientamento al sole degli individui a ritmo nictiemerale invertito, durante la "loro notte." *Boll. Ist. Mus. Zool. Univ. Torino*, **4**: 127.

PARDI, L., AND M. GRASSI, 1955. Experimental modification of direction-finding in *Talitrus saltator* (Montagu) and *Talorchestia deshayesei* (Aud.). (Crustacea-Amphipoda). *Experientia*, **11**: 202–205.

PARDI, L., AND F. PAPI, 1953. Ricerche sull'orientamento di *Talitrus saltator* (Montagu) (Crustacea-Amphipoda). I. *Zeitschr. vergl. Physiol.*, **35**: 459–489.

PITTENDRIGH, C. S., in press. "Circadian Rhythms and the Circadian Organization of Living Systems," in Cold Spring Harbor Symposia on Quantitative Biology, Volume XXV, Biological Clocks.

SEX DIFFERENCES AND ENVIRONMENTAL INFLUENCE ON DOPA-OXIDIZING ACTIVITY IN DROSOPHILA MELANOGASTER [1]

M. E. JACOBS [2]

Department of Zoology, Duke University, Durham, North Carolina, and Department of Biology, Bethany College, Bethany, West Virginia

Horowitz and Fling (1955) have described a rapid method for determination of tyrosinase activity in Drosophila. The method involves grinding adults, centrifuging, and decanting the supernatant as enzyme source. Colorimetric readings of the dopa-oxidizing activity are made to obtain enzyme-substrate reaction curves. The authors found that the maximal rate of dopachrome formation at 30° C. is directly proportional to enzyme concentration. This rapid method was used in a series of studies by the writer because it made possible quick repetition of determinations and thereby allowed for on-the-spot analysis of dopa-oxidizing activity during metamorphosis. (The exact nature of the enzymes involved is not known. Danneel (1943) has described three phenolases in Drosophila: one, a monophenolase which converts tyrosine to dopa, another a diphenolase which converts dopa to dopachrome, and a third which converts dopachrome to melanin.)

In attempting to apply this method, the writer obtained very different activity levels among 50-mg. samples of larvae and adults, even when these were taken from the same batch. It was found that modification of the method (as described below) eliminated some of the variation in activity rates between different samples. However, much variation still remained, and it was suspected that sex of the adults and larvae, and exposure of larvae to relatively dry air of the air conditioned laboratory were influencing the subsequent rate of dopa-oxidizing activity. The following study was undertaken to determine whether these factors do influence the rate of activity.

Materials and Methods

The flies used were those collected from a wild population at Beaufort, N. C. (see Jacobs, 1960). The strain is a light tan form, similar to Canton-S, made "isogenic" for chromosomes 1, 2, and 3 by means of a dominant inversion j90 marker stock from Dr. H. J. Muller and a C1B stock from Dr. M. Whittinghill.

The culture medium used was a modification of the Carpenter-Baker formula containing: 1000 cc. water, 40 g. white ground cornmeal, 15 g. agar, 40 g. dried Fleischmann's yeast type 2019, 1 g. KH_2PO_4, 8 g. Rochelle salt, 100 cc. white Karo syrup, and 0.5 g. of: NaCl, $CaCl_2$, $MnCl_2$ and $FeSO_4$. The medium was seeded with an aqueous suspension of dry active Fleischmann's yeast.

About 50 cc. of medium were placed in each half-pint milk bottle. To such

[1] This study was aided by the Lalor Foundation.

[2] Present address: Bethany College, Bethany, West Virginia.

bottles were added 30 female and 10 male parents. Each day, the parents were transferred to a fresh bottle to which ten fresh females and two males were added.

All cultures were kept at 25° C. in the dark. When the late larvae crawled up the sides of the bottles, as they do before pupation, the largest were removed and sexed, a few at a time, under water, on the basis of gonad size (see Bodenstein, 1950). Emergence tests of some of these larvae showed that the reliability of the sexing technique was excellent. For each enzyme determination, approximately 200 of the sexed larvae were placed in each of two 55 × 55 mm. specimen jars. One of these jars was placed, without a lid, in a 250-mm. Scheibler desiccator in which a saturated solution of $K_2CO_3 \cdot 2H_2O$ was kept in order to produce 43% R. H. (relative humidity) conditions (see Lange, 1939). The other jar was fitted with a lid with a hole 45 mm. in diameter cut in it and a disc of wire gauze fit in it, to prevent larvae from crawling out of the jar, which is possible when the walls are wet. This second jar was placed in a desiccator containing distilled water. The two desiccators were placed in the dark in a G. E. refrigerator kept at 24.5° C. with a Cenco 46042 heater thermostat unit. After the desiccators had stood for four hours, 50 mg. of the largest larvae (to the nearest whole larva) were used for each enzyme determination.

Adults were obtained by etherizing them within an hour after emergence from the pupa cases, while they were still translucent and just after expansion of the wings. Before the adults were used for the colorimetric enzyme determinations, the heads were removed so the eye pigments would not interfere with the tests. It took about five minutes to remove the heads from the 50 mg. of flies used in each determination. The flies were weighed before removal of the heads.

The method for dopa-oxidizing activity was as follows: from the 100% R. H. desiccator were weighed 50 mg. of larvae (to the nearest whole larva). These were washed with distilled water into a powder funnel, to the bottom of which was fitted a wire gauze. The funnel was then inverted over the open end of a 13 × 100 mm. Corning #7725 tissue grinder tube sawed off to a length of 45 mm. so it would fit the high speed head of the centrifuge. Distilled water was streamed onto the wire gauze to wash the larvae into the grinder tube. This water was pipetted out and replaced three times, after which all free water was pipetted from the inside of the tube, and the outside was wiped dry. The tube with the larvae was then weighed. The larvae were ground in the tube in 1 ml. of phosphate buffer (pH 6). To the tube was immediately added 0.5 ml. of L-dopa (0.02 *M* aqueous), and the tube was placed in a water bath at 30° C. for ten minutes. The tube was then placed into ice water for one minute while 1 ml. of chloroform was added. The tube was then removed, Parafilm was held over the open end, and the tube was shaken a few seconds so the lipids would dissolve in the chloroform, for lipids were found to cause turbidity which interfered with colorimeter readings. The tube was then placed into the high speed head of the centrifuge (0° C.) and brought to 6,000 *g* in one minute, left there for five minutes, then reduced to stopping in one minute. Into a ½-inch tube of the B & L Spectronic 20 colorimeter were placed 0.5 ml. of the supernatant and 2 ml. of the phosphate buffer. A reading was taken against a distilled water standard at 475 millimicra, and the tube was placed in the water bath at 30° C. for ten minutes, after which another reading was made.

An equal number of larvae were removed from the 43% R. H. desiccator and treated as were the 100% R. H. larvae except that after the tube with the larvae was weighed, water was added to bring the total weight up to that shown by the tube with the 43% larvae. (In the study of unsexed larvae kept under the two conditions as described in the next paragraph, in which the larvae were never in water, the 43% R. H. larvae were found to weigh an average of 0.833% less than the 100% R. H. larvae.) Adults were added unwashed to the grinder tube, ground in buffer, and treated the same way as were the larvae.

When it was found that larvae at 43% R. H. showed increased dopa-oxidizing activity as compared with those at 100%, the question arose as to whether the 43% larvae would also pupate at a faster rate. To test this, 200 unsexed larvae were placed in the jar in each desiccator and allowed to remain for four hours as before, after which 20 of the largest larvae in each desiccator were weighed, boiling water was poured into the jars, and the pupae were counted. This was repeated ten times for the four-hour period and ten times for an eight-hour period.

Results

The results of the tests showed larvae kept at 43% R. H. had greater dopa-oxidizing activity than those kept at 100% (Table I). For the larvae at 43%,

Table I

Dopa-oxidizing activity of Drosophila melanogaster *as indicated by colorimeter readings of reactant mixtures of tissue homogenates and dopa substrate. A low per cent of transmittance indicates a high rate of dopa-oxidizing activity*

Type of flies	Number of 50-mg. samples	Per cent of transmittance		
		Reading no. 1 (after centrifuging) Mean ± S.E.	Reading no. 2 (10 min. after no. 1) Mean ± S.E.	Difference between no. 1-no. 2 readings Mean ± S.E.
Larval females in humid conditions (100% R. H.)	15	70.86 ± 2.42	59.66 ± 3.47	11.20 ± 1.63
Larval females in dry conditions (43% R. H.)	15	60.00 ± 2.06	42.40 ± 3.04	17.60 ± 0.92
Larval males in humid conditions (100% R. H.)	13	76.07 ± 2.77	66.61 ± 2.45	9.46 ± 1.83
Larval males in dry conditions (43% R. H.)	13	66.38 ± 3.58	51.61 ± 3.97	14.77 ± 1.58
Adult females	53	59.77 ± 0.97	51.67 ± 1.24	8.10 ± 0.52
Adult males	38	85.60 ± 0.23	84.00 ± 0.34	1.60 ± 0.71

the colorimeter readings taken just after centrifuging, as well as those taken ten minutes later, showed lower per cent of transmittance than did those for the larvae kept at 100% R. H. Since this drop in per cent of transmittance is sufficient to show the relative activity rate, this figure alone will be considered in this discussion for the sake of simplicity, and will be referred to as activity in colorimeter units.

Larval females at 100% R. H. showed 11.20 units, while those at 43% R. H. showed 17.60 units of activity. Larval males at 100% R. H. showed 9.46 units, while those at 43% showed 14.77 units. Adult females showed 8.10 units, while adult males showed only 1.60 units. It is thus observed that the larvae at 43% R. H. showed greater activity than those at 100%, and adult females showed greater activity than adult males. All these differences are significant well below the 5% level. It appears that larval females show greater activity than larval males, but the observed "t" value for this difference is small (1.52) and above the 5% level of significance.

For both larvae and adults, the average wet weight of females was greater than that of males (Table II). It took an average of only 25.52 larval females to weigh 50 mg., while it took 30.53 males. Likewise, it took only 39.87 adult females, while it took 55.14 males.

In the counts of larvae kept at 100% and 43% R. H. for four hours, there were 381 pupae in the 100% jars and 352 in the 43% jars. The chi-square value for this difference is only 1.147, which is not significant. More significant results were obtained in the eight-hour samples, in which 674 pupae were counted in the

TABLE II

Mean number of flies in 50-mg. samples taken to the nearest whole fly. The same number of larvae were used in the 100% and 43% relative humidity samples

Type of flies	Number of 50-mg. samples	Number of flies per 50-mg. sample (Mean ± S.E.)
Larval females	15	25.52 ± 0.06
Larval males	13	30.53 ± 0.05
Adult females	53	39.87 ± 0.07
Adult males	38	55.14 ± 0.09

100% jars and only 594 in the 43% jars. The chi-square value for this difference is 50.473, which is significant below the 1% level. Thus, contrary to what was expected, the larvae at 100% R. H. pupated at a faster rate than those at 43% R. H.

DISCUSSION

The observation that *D. melanogaster* females show more dopa-oxidizing activity than males raises the question as to whether this activity is involved in general metabolism, perhaps as a result of egg production. This would seem reasonable in view of the fact that differences in gonad size, as observed among male and female larvae, are less than the difference in size between the reproductive structure (egg masses) of the adult female as compared with the small mass of the adult male testes: and difference in dopa-oxidizing activity among male and female larvae is also smaller than the difference in activity between male and female adults. Bodine and Allen (1938) have demonstrated, in grasshopper eggs, a tyrosinase proenzyme which can be activated by shaking and other means. Kucera (1934) has found *D. melanogaster* adult females to consume more oxygen than adult males. However, Sussman (1952) found no difference in oxygen uptake in *Platysamia cecropia* moth diapausing larvae injected with tyrosinase inhibitors, as compared with controls, and he suggested that tyrosinase is not a terminal oxidase in those larvae.

The increased rate of dopa-oxidizing activity in the larvae at 43% R. H. might be due to control by Weismann's ring gland, which in turn is regulated by the nervous system. Dennell (1949) has inhibited tyrosinase activity in *Calliphora erythrocephala* by destruction of the gland. In this fly, in spite of the presence in the blood of both enzyme and substrate, no oxidation of tyrosine takes place until the pupation hormone controlled by the gland is liberated. Dennell discovered a glucose dehydrogenase system which declined in activity as the larvae approached pupation, while tyrosinase activity increased. Dennell suggests that the dehydrogenase system is controlled by the gland and inhibits tyrosinase activity.

The classical view of the role of phenolase activity in insects is that certain phenolic oxidation products of tyrosine are responsible for tanning and hardening of the larval cuticle to form the puparium. (See Dennell, 1958a, for review.) It was this theory which prompted the present study in which pupae were counted following killing with boiling water. It was thought that the increased phenolase activity would cause increased rate of puparium formation. But, contrary to what was suspected, the larvae at the lower humidity (43%), in which dopa-oxidizing activity was accelerated, showed a slower pupation rate than those at 100%. This decreased rate at lower humidity has not yet been explained.

The failure to find an increase in pupation rate to accompany an increase in dopa-oxidizing activity in larvae at the lower humidity is in accordance with the finding of Dennell (1958b) that inhibition of tyrosinase by injection of phenylthiourea into mature larvae of the fly, *Calliphora vomitoria*, did not modify puparium formation. Dennell (1958a) has been led to the view that the dihydroxyphenol formed in the blood of insects by the specific action of tyrosinase on tyrosine is not of primary importance in giving rise to a tanning quinone. He thinks this activity is of importance only in the formation of melanin, although he admits the possibility that some dopa formed in the blood may be hydroxylated in the cuticle to yield a slight supply of the tanning quinone in addition to that produced by the major source. Dennell thinks the major source of the tanning quinone arises from non-enzymatic hydroxylation of phenylalanine and tyrosine to hydroquinone, which is converted to para-benzoquinone which tans the cuticle. Malek (1957) also observed that sclerotization and melanization are two separate processes in the desert locust.

Ohnishi (1954a) found that ebony larvae of *D. melanogaster*, in which the pupa case is lighter than in the wild type, showed less tyrosinase activity than did the wild type. He also found (1954b) in larvae of ebony *D. virilis*, in which the pupa case is darker than in the wild type, that tyrosinase activity was higher than in the wild type. Ohnishi (1953) also found tyrosinase activity in *D. melanogaster* larvae increases five to six hours before puparium formation, that is, nearly at the critical period of pupation hormone. These observations lead Ohnishi to the view that tyrosinase is responsible for the color of the pupae. However, Danneel (1943) found no difference in tyrosinase activity among ebony, yellow, black, and wild type *D. melanogaster*, but he did note varietal differences. Karlson and Schmid (1955) found a metamorphosis hormone in Calliphora larvae to cause browning of the cuticle, and this browning was said to be due to tyrosinase, which the authors extracted. Much additional work is needed before this question of the role of tyrosinase and its genetic control in insect metabolism can be elucidated.

SUMMARY

1. Colorimetric determinations of dopa-oxidizing activity in newly emerged adults and late larvae close to pupation time showed adult *Drosophila melanogaster* females to have higher activity rates than adult males. This may also be true for larvae, but the difference here was slight. The larvae showed higher rates than adults.

2. Larvae kept four hours at 43% relative humidity (R. H.) showed higher rates of dopa-oxidizing activity than did those kept at 100% R. H.

3. Contrary to what was expected, larvae kept at 100% R. H. pupated at a faster rate than those kept at 43%.

4. Female larvae and adults were found to be heavier than males.

5. The postulated role of tyrosinase in puparium formation is discussed.

LITERATURE CITED

BODENSTEIN, D., 1950. The postembryonic development of Drosophila. *In:* Biology of Drosophila, Edit. by M. Demerec, pp. 275–364. New York: Wiley.

BODINE, J. H., AND T. H. ALLEN, 1938. Enzymes in ontogenesis (Orthoptera) IV. Natural and artificial conditions governing the action of tyrosinase. *J. Cell. Comp. Physiol.*, **11**: 409–423.

DANNEEL, R., 1943. Melaninbildende Fermente bei *Drosophila melanogaster*. *Biol. Zentralbl.*, **63**: 377.

DENNELL, R., 1949. Weismann's ring and control of tyrosinase activity in the larvae of *Calliphora erythrocephala*. *Proc. Roy. Soc. London, Ser. B*, **136**: 94–109.

DENNELL, R., 1958a. The hardening of insect cuticles. *Biol. Rev.*, **33**: 178–195.

DENNELL, R., 1958b. The amino acid metabolism of a developing insect cuticle; the larval cuticle and puparium of *Calliphora vomitoria* III. The formation of the puparium. *Proc. Roy. Soc. London, Ser. B*, **149**: 176–183.

HOROWITZ, N. H., AND MARGUERITE FLING, 1955. The autocatalytic production of tyrosinase in extracts of *Drosophila melanogaster*. *In:* Amino Acid Metabolism, Edit. by W. D. McElroy and B. Glass, pp. 207–218. Baltimore: The Johns Hopkins Press.

JACOBS, M. E., 1960. Influence of light on mating of *Drosophila melanogaster*. *Ecology*, **41**: 182–188.

KARLSON, P., AND H. SCHMID, 1955. Über die Tyrosinase der Calliphora-Larven. *Hoppe-Seyler's Zeitschr. Physiol. Chem.*, **300**: 35–41.

KUCERA, W. G., 1934. Oxygen consumption in the male and female fly, *Drosophila melanogaster*. *Physiol. Zoöl.*, **7**: 449–458.

LANGE, N. A., 1939. Solutions for maintaining constant humidity. Handbook of Chemistry, p. 1181. Sandusky, Ohio: Handbook Publishers, Inc.

MALEK, S. R. A., 1957. Sclerotization and melanization: two independent processes in the cuticle of the desert locust. *Nature*, **180**: 238.

OHNISHI, E., 1953. Tryosinase activity during puparium formation in *Drosophila melanogaster*. *Japan. J. Zool.*, **11**: 69–74.

OHNISHI, E., 1954a. Melanin formation in the mutant "ebony" of *Drosophila melanogaster*. *Annot. Zool. Japon.*, **27**: 76–81.

OHNISHI, E., 1954b. Tyrosinase in *Drosophila virilis*. *Annot. Zool. Japon.*, **27**: 33–39.

SUSSMAN, A., 1952. Tyrosinase and the respiration of pupae of *Platysamia cecropia* L. *Biol. Bull.*, **102**: 39–47.

AN EXPERIMENTAL DEMONSTRATION OF ECHO-LOCATION BEHAVIOR IN THE PORPOISE, TURSIOPS TRUNCATUS (MONTAGU)[1]

KENNETH S. NORRIS, JOHN H. PRESCOTT, PAUL V. ASA-DORIAN AND PAUL PERKINS

Department of Zoology, University of California, Los Angeles 24, California, Marineland of the Pacific, Palos Verdes Estates, California, and U. S. Navy Electronics Laboratory, San Diego, California

Many people have suspected that cetaceans use echo-location as a means of navigation. A considerable body of evidence has been marshalled on the subject, some of which shows that this is actually the case.

First, field observations made by persons trying to catch these remakable animals have shown that, not only are they far more difficult to catch than one might suppose, but also that they can avoid nets or blind channels when vision, as we know it, would seem to be ruled out (McBride, 1956; Norris and Prescott, in press).

Second, a variety of sounds are known to be produced by cetaceans, in particular by odontocetes, including some of extremely high frequencies (Wood, 1954). Further, certain of these sounds are regularly emitted when these animals locate objects in test situations where vision was kept to a minimum (see for example, Schevill and Lawrence, 1956, and Kellogg, 1958).

Third, the cetacean middle and inner ear, and the acoustic portions of the brain have been shown to be capable of the reception, binaural isolation and interpretation of high frequency sounds such as might be used in echo-location behavior (Haan, 1957; Fraser and Purves, 1959). In fact, this acoustic equipment is probably second to none in the animal kingdom, including that of the Microchiroptera.

Fourth, observations of behavior and concurrent acoustic recordings have been made using bottlenose dolphins, usually held in extremely turbid tanks, which showed that these animals were able to find their way among obstacles with little trouble, even on dark nights. Taken together these demonstrations indicate echo-location behavior with a reasonable degree of assurance, particularly since trains of high-frequency sound bursts were usually demonstrated to be emitted by the animals during solution of these problems. In none of these experimental studies was sight completely eliminated. Though water in the test tanks was extremely turbid, and human sight would not have served for more than 10 to 30 inches, still the experimenter's view of the animal was necessarily poor and it is not inconceivable that an unknown factor or factors might sometimes have directed it until sight could have played a part. For example, acute sound localization could have produced much of the behavior that has been noted. It is quite conceivable that when a test animal hears the sound of a reward fish splashing into the water,

[1] Contribution Number 12, Marineland of the Pacific Biological Laboratory.

it is able to orient to the splash, swim toward the sound source, and navigate the last few feet or inches by sight.

This sort of explanation, however, will not explain behavior in which the porpoise quickly located a reward, time after time, which was silently presented at randomized locations beneath the water's surface. It will not explain the simple experiment in which water droplets were released onto the surface of the muddy water from a height, causing immediate orientation of the porpoise but no searching behavior, after similar tests with BB shot, rocks and fish caused both orientation and searching behavior (Kellogg, 1958).

Likewise, vision seems to have been ruled out in the size discrimination test in which a bottlenose porpoise was required to choose between two species of fish of different sizes, one of which was acceptable as food and the other inacceptable. The choice was considered to be made when the test animal touched its snout to one of the target fish. The tank water was so murky that human divers could not make the discrimination when at a point equal to the distance from the porpoise's eye to the tip of its snout (Kellogg, 1959).

Since the study presented here is an extension of these previous experimental efforts, the most important ones will be described briefly in chronological order. Kellogg and Kohler (1952) transmitted sounds into a clear display tank at Marine Studios, Florida, and were able to note variations of swimming behavior that indicated hearing by captive bottlenose porpoises up to a frequency of about 80 kc. However, it seems doubtful that these observations defined upper limits for hearing since the reliability of their transducer at these high frequencies has been questioned (Schevill and Lawrence, 1953), and since it is probable that the behavior elicited by the sounds was a fright response. While the animals may have heard higher frequencies than 80 kc they need not necessarily have been frightened by them. The work was important, however, because it was suggestive of hearing capability more than four times as high as the upper limit for the average human. A more comprehensive report by Kellogg (1953) demonstrated a similar response from three separate groups of bottlenose porpoises. The upper sound level at which the response was produced 100% of the time varied from 50 to 80 kc.

Kellogg, Kohler and Morris (1953) studied the frequency spectrum of a porpoise click or pulse. They found that the majority of energy was concentrated between 20 and 30 kc. An interesting discussion of the possible utility of these sounds as echo-location signals is included. They note that such sounds become highly directional at higher frequency levels. The shorter wave-lengths also would permit greater resolution than lower pitched signals, as well as being above the majority of background noise in the sea.

Schevill and Lawrence (1953) used the conditioned response technique to determine an upper frequency limit for hearing in *Tursiops truncatus*. Their captive, held in a murky pond, was required to come to their hands for a reward upon hearing a sound signal generated in the tank. The response dropped from 100% success at 120 kc to 13% at 153 kc, the upper limit of their sound generator.

In spite of hours of listening and recording sounds, these authors reported that their test animal was able to navigate without difficulty around its tank, when not feeding, apparently without emission of any sounds at all. However, they stated

that the background noise level of their test tank was "deplorably high," primarily from snapping shrimp living in the bottom mud.

In a later study these same authors (Schevill and Lawrence, 1956) reported on observations made in a quiet pond in which they found that the porpoise emitted (p. 4) "a characteristic sequence of 'creaks' as he came in for a fish and that in the last meter or so these sounds were matched to 'horizontal movements of his head.'" These sounds contained both audible and high frequency components and were associated with food location by the animal. The sound intensity was thought to be low enough so that these clicks could have been masked by the shrimp in the previous study. More significant still was the demonstration that the porpoise could locate a reward fish slipped into the water at randomized positions on either side of a net stretched in the tank. These tests represent the first experimental demonstrations of the presence of echo-location behavior in the species *Tursiops truncatus*.

Kellogg (1958, 1959) performed an extensive series of tests that provide additional evidence for echo-location behavior in the bottlenose porpoise. His test animals were able to discriminate between two gates, one closed by a plexiglass sheet, and the other open, presented in a randomized fashion. The animals invariably went through the open gate. His animals were also able to navigate through a maze of poles hung in the turbid tank, pluck a fish from an open window while ignoring one held behind glass, and as has been discussed before, distinguish between an object dropped into the water, as opposed to a measured amount of water dropped on the surface, and discriminate between different sizes of fishes suspended in the water.

Heel (1959) investigated the directional hearing capability of the harbor porpoise, *Phocoena phocoena*. He found a minimum or threshold angle of discrimination between two 6-kc tone generators to be 8° on either side of the sagittal plane of the animal's head. This figure increased somewhat with a lowering of the frequency of the signal. He states that these results are comparable with those found for humans, considering the almost five-fold increase in the speed of sound in water as compared to that in air.

Vincent (1960) has studied the sound emissions of the Atlantic saddle-back or common dolphin (*Delphinus delphis*). Three kinds of sounds are reported: whistles, popping or crackling sounds, and mewings. The popping sounds proved quite similar to the echo-location signals of *Tursiops truncatus*. Vincent was unable to record sounds above about 15 kc because of the limitations of his equipment so the entire frequency range covered by the sounds remains unknown.

The classical test of complete suppression of vision while leaving the sound emission and reception equipment intact still needed to be performed. If in this test the animal retained its ability to navigate and locate objects, we could then conclude that sight was not essential to orientation and navigation in this animal. Finally, if either the sound-sending or -receiving apparatus could be impaired, and if disorientation resulted, still in the absence of vision, then we could conclude that sound was essential to navigation, and hence that echo-location behavior did indeed occur.

We set out to attempt these tests at Marineland of the Pacific during July of 1959. Our tests continued until February, 1960, when the test animal was re-

quired for show purposes and our study had to be terminated. Since there is little likelihood of an early resumption of these experiments, some incomplete tests are reported here and are of qualitative value only.

Materials and Methods

Our experimental subject, a young adult female Atlantic bottlenose porpoise of the species *Tursiops truncatus*, was kept in a circular reinforced concrete tank 35 feet in diameter and 5½ feet deep. Our first attempt was to train this animal to take a blindfold that would completely eliminate her vision while leaving hearing and sound transmission unimpaired.

Training

The porpoise, named Kathy, was slightly trained at the time we obtained her for our tests. Our initial step was to teach her to take cut fish rather than whole ones. This allowed us to demand a much greater number of responses for a given weight of food than could be obtained with whole fish. Thoughout the tests her diet consisted of fresh frozen Pacific mackerel (*Pneumatophorus diego*), fortified periodically with crude aureomycin and complex vitamin capsules. She was given from 12 to 15 pounds per day, depending upon her condition and drive.

Once Kathy began taking cut fish readily, she was trained to take the pieces of fish from our hands. Then we required her to allow us to touch the tip of her rostrum with the fingers of one hand before the reward was given with the other.

Throughout the tests a bridging stimulus was used to provide immediate reinforcement of desirable reactions. This consisted of a blast from a police whistle given as soon after the performance of a desired behavior pattern as possible. She soon learned that when she heard this whistle she had done what was required and would then receive her reward. In this manner we were able to reinforce actions performed across the tank or far out of reach in mid-tank.

Our plan was to use the process of multiple approximations in simulating the placement of a blindfold, step by step, until the actual act was performed. It developed that Kathy was very easily disturbed by any object that touched the areas around her eyes and blowhole, and that these approximations could be made only after many halting steps.

The first approximation was to slide one hand down her rostrum, step by step, until the fingers of one hand finally rested over one eye. In about two weeks she allowed us to hold one cupped hand over one eye for a few seconds without objection. The second approximation consisted of working both hands together into this same position, thus covering both eyes.

Once this was accomplished it was decided to approximate the blindfold itself by wearing cotton gloves during the eye-covering operation. This proved to be one of the most difficult steps in the training procedure. The animal panicked at the touch of the foreign material and training had to be redone, nearly anew. Retraining after such a balk by the animal was, however, much more rapid than when first attempted.

The first actual blindfold that was contemplated was a pair of eye cups, to be held in place by an overhead spring-steel loop, which would exert a moderate and

FIGURE 1. Latex suction cups being applied over the porpoise's eyes. The animal has been given a signal to come to the operator and to station herself in front of him. Note that she closes her eyes before the cups are pressed in place.

steady pressure. As an approximation of this type of blindfold, a heavy loop of tygon tubing was slipped into the gloves as far as the thumbs, curving between the operator's hands. The animal objected rather strenuously to this approximation, fleeing at first at the slightest touch of any of the tube in the region of her blowhole. Once she became reasonably accustomed to this treatment, a series of actual blindfolds built on this principle were tried, all without significant success. The animal refused to breathe while these were in place, and quickly shook them off with violent movements of her body.

After two months' work we finally stopped attempts with this type of blindfold, both because of the sensitivity of the animal to the overhead loop and because we thought it possible that the loop might cause turbulence in the vicinity of the ears and the nasal sac system that could produce erroneous results. Instead, we decided to construct a pair of latex suction cups that would just cover the animal's eyes, leaving the ear region undisturbed, and which would be without the overhead loop. This was done and after a number of trials with different cup designs a pair was developed that stayed on quite well during normal swimming movements (Fig. 1).

In the first test with these cups in place the animal swam from the operator's hands and circled the tank without any hesitation whatsoever. Never, during this or subsequent tests with the blindfolds in place, did Kathy show any tendency to bump into any obstacle in the tank. Her swimming was normal, both as to speed and maneuverability. The only detectable differences from her swimming with sight were fairly regular "rapid peering" movements with her head and neck, when she wished to "inspect" some part of her enclosure. This sort of sound-scanning has been described by both Schevill and Lawrence (1953, 1956) and Kellogg (1958, 1959).

The usual routine when testing the animal was as follows: The operator slapped the palm of his hand against the tank wall above the water line several times in succession. Kathy learned that this was her cue to come to him for placement of the blindfolds. The operator then placed his hands in the water

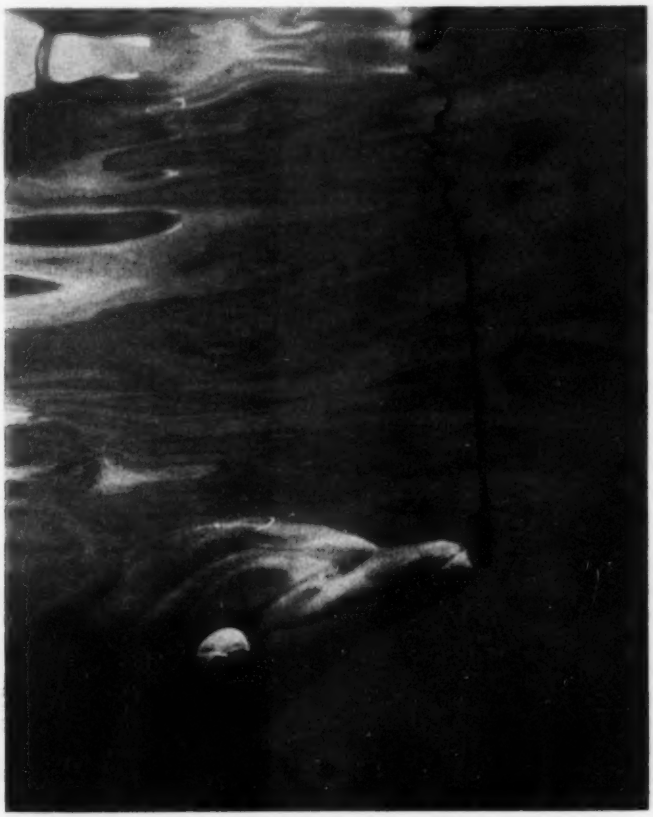


FIGURE 2. The porpoise pressing the bell target with the tip of her rostrum. Photograph courtesy of Richard Hewett.

with the cups held in his palms. She swam between his hands and permitted him to press the cups over her eyes. She always closed her eyes just before the cups were applied (Fig. 1). Then she was cued to perform the specific act required of her, by a variety of sound cues. In the case of the major test two bursts from an 18-kc subsurface transducer cued her to swim around the tank and locate a target suspended below the water surface from a randomly chosen location on the tank rim, swim to it, press it with the tip of her rostrum, thus ringing a bell, and return to the operator for a reward (Fig. 2). When recordings of her sound emission were made the reward was generally drifted in the water at the desired distance from the hydrophone. In this way she could be made to run in on the hydrophone while attempting discrimination of the small reward object.

Results

A detailed analysis of sounds recorded from Kathy will not be presented here. However, there are certain observations, complete in themselves, that seem pertinent to this report.

Cruising sounds

When Kathy cruised blindfolded about the tank, not searching for a specific target object, she emitted sporadic sound pulses of the type used in echo-location. As a general rule these sounds probably kept her informed about the confines of her tank and little more. These sounds varied in number from single pulses to short trains of about six pulses. The length of each pulse varied from about 1 millisecond to 1.5 milliseconds. The repetition rates of these sounds were extremely variable. We noted that these cruising sounds were emitted by Kathy even when she was *without blindfolds*, swimming in crystal-clear water. The physical characteristics of the individual bursts of sound agree well with the clicks recorded and analyzed by Kellogg, Kohler and Morris (1953).

Searching sounds

After Kathy pressed the target while blindfolded, and rang the bell, she then returned across the tank for her reward. While this was dropped in front of the hydrophone she first reacted to the initial splash as the fish fragment (usually about a 2-inch cross-section of a mackerel) hit the water, oriented toward it, and then swam directly to it and picked it up as it drifted downward. Scanning movements by the animal usually began 12 feet or more from the reward, particularly if she failed to orient properly to the initial splash. These scanning movements became marked, usually when the animal was about three feet from the reward, sometimes becoming quite extreme as she came closer. This scanning consisted of jerky movements of the head involving lateral swings through as much as 25–30° of arc, or of circular movements of the rostrum that often caused the animal to bend and rotate her neck rather acutely. These scanning movements were particularly marked if the animal was required to pick up a reward fragment drifted very close to the hydrophone. Discrimination between two such closely adjacent objects obviously required much sound scanning, but even if the two were less than an inch apart the fish usually was picked very delicately from the

water without touching the hydrophone. The fact that other investigators usually have not observed such extreme searching movements may have been due to the presence of some vision at close range in their experiments. The only case in which such exaggerated movements are reported is that observed by Schevill and Lawrence (1956) when their animal temporarily became virtually blind in both eyes. They state (p. 12): "Once when he swam past a fish in the water, he worked his way back to it slowly with very exaggerated head noddings and took the fish awkwardly deep in his mouth."

Pulse repetition rate during the initial orientation of the porpoise to her reward was variable but usually fairly low. The lowest rate observed at this time was 16 pulses per second. As the animal approached the fish this rate increased in a semi-systematic way, as a series of trains of sounds, usually reaching about 190 pulses per second just before the reward was taken. Then, the rate usually decreased abruptly to 26–50 pulses per second just as the fish was taken. Then as Kathy swerved away from the hydrophone and the wall with the fish in her mouth she often gave out a burst of pulses at extremely high repetition rates, the maximum observed being 416 per second.

Reverberations

During tests in which Kathy was required to locate a reward dropped in front of a submerged hydrophone, our recordings show as many as seven reverberations of each of her outgoing pulses between each two pulses in a train of sounds. These reverberations of her signal came from the surface, the bottom, the walls and objects suspended in the tank. In spite of this cacophony Kathy was able to locate the fish fragment and to swim directly to it. No attempt was made to play her own signals back to her.

Directionality of the sound beam

We noted that when the porpoise approached the fish fragment reward and began scanning movements with her head we began to hear marked fluctuations in the volume of the audible signal from our amplifying system.[2] This same phenomenon was also noted by Schevill and Lawrence (1956). Such directionality of the sound has been demonstrated for bats (see Griffin, 1958, p. 104). These changes in sound intensity could be correlated visually with movements of Kathy's head. When she pointed her head slightly downward so that her melon and the dorsal surface of her rostrum were in a direct line with the hydrophone, the volume increased. When she raised her snout so that the ventral surface was on a direct line with the hydrophone, the volume decreased markedly. A diver submerged in the tank with Kathy when she was blindfolded reported that the intensity of her audible signal was greatest when her rostrum was pointed directly at him, and decreased when she swung her head to the side.

[2] During most of these experiments the sound pick-up and recording system used consisted of a U. S. Navy Model AN/PQM-1A sonar set, which covers a frequency band of 30 cycles through 33 kilocycles. The hydrophone, which contains its own preamplifier, was periodically calibrated during the tests. The system is omnidirectional and although push-button filters are an integral part of the receiver, for purposes of this study all signals were recorded in the open position.

A simple test was performed to obtain some idea of the configuration of this putative sound beam. This test consisted simply of drifting pieces of fish around the blindfolded porpoise, in various positions relative to her head, at the same time the reward signal was given. Even though Kathy was stationed virtually motionless in the water in front of the operator when these tests were made, the sound pick-up system revealed that she was emitting characteristic echo-location sounds.

Briefly, the results of these tests were that she was able to detect and snap up any fish that descended from above and ahead of her melon, while she invariably lost those that drifted below the level of her rostrum, even though they were sometimes within less than an inch of her jaws (Fig. 3). Her only successes in obtaining reward fragments drifted below her jaws came when, during her scanning

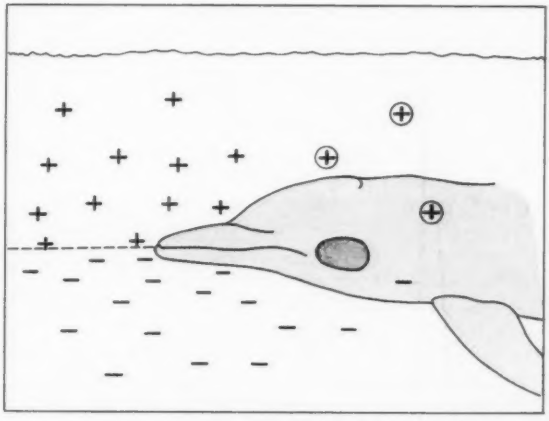


FIGURE 3. A diagram showing the detection of fragments of fish drifted into the water near the blindfolded porpoise. Plusses indicate the positions where food fragments were detected immediately and snapped up without hesitation. Minuses indicate positions at which reward fragments were lost. Circled plusses indicate positions at which the porpoise did not immediately snap up the reward, but returned and recovered it on another pass.

movements, she bent far enough downward so that the fish was in line with the horizontal axis of her rostrum, or above it.

Fish drifted lateral to her head were picked up as far back as her eye, as long as they were above the level of her jaws. Fish drifted down on top of the anterior apex of her melon were located with no trouble. Fish drifted down above her head in the vicinity of her blowhole were usually picked up when she turned and made a second pass at them as they drifted toward the tank bottom.

Thus it appears that the animal probably sends, and may also receive, sound in a directional fashion during echo-location. It also appears that the site of this directionality is localized, at least in part, in the region of the upper jaw and melon. The observations by the diver, and those of the authors who could correlate sound intensity with the position of Kathy's head in relation to the

hydrophone, indicate that the upper jaw and melon region is connected with beamed sound transmission though they do not preclude the possibility of sound reception at the same site. The echo-location system of the bottlenose porpoise therefore does not appear to function solely on the basis of an omnidirectional sound field emanating from a localized or point source as has been assumed in the past. In view of the wide range of frequencies present in individual sound pulses it seems likely that the degree of directionality may vary systematically with frequency. One bit of evidence gained during our experiments tends to support this view. A small especially-constructed hydrophone, sensitive to a narrow band of frequencies centered around 100 kc, was lowered into the tank. The only time signals from this hydrophone could be picked up on our oscilloscope was when Kathy pointed her rostrum accurately at the hydrophone.

Experimental interference with the sound-sending and -receiving equipment

The lack of dependence upon vision is amply demonstrated by the blindfold experiment. While the sound emission and associated behavior is strong evidence for echo-location in the blindfolded animal, the crucial test is still disruption of the sound path of the blindfolded porpoise in some way, coupled with the results of such disruption. The first approach to this problem was simply to cover the external ear openings with a patch of material that would disrupt the passage of sound. In this case two small suction cups were used, each 1 inch in diameter and lined with ⅛ of an inch of foam neoprene sheeting. Such foam neoprene forms an excellent acoustic mismatch with water because of its multitude of tiny trapped gas bubbles. The porpoise did not object to having these cups pressed in place over her external ear openings, and then navigated perfectly with both eye and ear cups in place. This result is, however, considered inconclusive for two reasons. First, it was impossible to tell how firmly the neoprene liner of the cups was pressed against the skin of the animal at the periphery of the neoprene patch. Conceivably there could have been places in which the barrier consisted of no more than the latex of the cup and the thin border of the neoprene patch. Thus there would have been no effective sound barrier. Second, if odontocetes rely upon transmission of sounds through the blubber and flesh to the tympanum and middle ear ossicles, as has been recently postulated (Haan, 1957), no disorientation would have been expected. According to this theory the external auditory canal is a rudiment. Support for this idea is provided by the discovery that in at least one species of porpoise (*Phocoenoides dalli*) the canal does not reach the surface of the skin (Norris and Prescott, in press).

Because of these uncertainties we turned to another approach. A mask of ¼" neoprene sheeting was constructed that covered Kathy's entire melon and upper jaw, including its tip. We hoped to train the animal to wear this mask while blindfolded. If the sound beam, presumed to emanate from this region, was actually essential to her blind navigation she should then manifest disorientation in some way when wearing this mask. As a means of attachment the mask was equipped with latex suction cups at appropriate points and was held on her jaws by means of rubber bands. Although Kathy was relatively docile and continued to come to her trainer upon command, we were never successful in making her wear this mask for more than a few seconds. On the few occasions when it was

attached properly to her head and jaws, she backed away from the trainer out into the open water of the tank and shook her head violently until the mask was shaken loose. Then, in a spirit of cooperation with her trainers, she sometimes picked up the floating mask and carried it back to them. This friendly but adamant refusal to wear the mask continued for two months until the termination of our tests. During this time she gave not the slightest hint that she would eventually submit to wearing the mask. During this entire time she never swam forward with the mask in place.

While her behavior during these mask tests did not give clear-cut evidence of disorientation it may be that the refusal we observed was the only kind of behavior we could expect from a highly intelligent animal faced with the loss of her visual and auditory sensory windows.

The discrimination ability of a blindfolded porpoise

Several tests, all incomplete, were performed to test the refinement of Kathy's echo-location ability.

First, two lines on pulleys were stretched across Kathy's pool, 10 feet apart From each of these lines one-inch diameter iron pipes were suspended at four- to seven-foot intervals. Each pipe reached nearly to the bottom of the tank. Since the lines could be moved independently, a maze of variable characteristics could

FIGURE 4. The porpoise maneuvers between two maze poles.
Photograph courtesy of Richard Hewett.

be produced. Kathy was run through this maze several times daily for a period of nearly two months, and only once did she touch a pole. Her ability to snap up a reward fish fragment dropped inches from a pole was remarkable. She would often circle the pole near which the reward had been dropped, bend her neck sharply, and delicately pluck the fish out of the water as it sank. At the same time, she avoided hitting the poles or the wall behind her with the utmost finesse, when her flukes and tail stock swung around (Fig. 4). Her one contact with a pole seemed to be a deliberate sidewise slash with her tail flukes, as she passed between two poles.

This test differed from that reported by Kellogg (1958) in that the poles of the maze were smaller (one inch in diameter as opposed to triangular poles two inches on a side), and because the poles were often much closer together. The poles in Kellogg's maze were eight feet apart.

The ability of porpoises to discriminate between objects of different sizes by sound has been shown by Kellogg (1959). It seems probable from the following test that discrimination is not limited to size differences alone but extends to objects of the same size but of different sound-reflecting characteristics.

A 1½" long veterinary gelatine capsule (½ ounce capacity) was filled with water and carefully weighted with small pebbles so that it would sink slowly when placed in water. Then a piece of mackerel flesh was cut the same size as the capsule. When both were thrown simultaneously into the tank and the reward whistle signal sounded, blindfolded Kathy approached both slowly sinking objects, scanned both with swings of her head, and without hesitation snapped up the mackerel fragment. This test was repeated several times with the same results. She never gave the sinking capsule more than a passing inspection. It should be noted that the mackerel fragment was cut entirely from muscle and skin and did not contain portions of the swim bladder or other gas filled spaces.

Discussion

This demonstration of navigation and discrimination by a blindfolded bottlenose porpoise provides a tool for the study of cetacean echo-location that, if used further, should allow direct attack on several of the most vexing problems involved in this navigation system. In particular, refined work on thresholds of discrimination by sound should now be relatively simple. Also, it should be possible to define accurately the characteristics of the sound field emitted by a cetacean, using a trained blindfolded animal. Perhaps such a trained animal could be used to explain the most obscure problem of all—that of the actual mechanism by which the porpoise uses trains of sound pulses to "inspect" its environment.

Summary and Conclusions

1. A method was devised whereby an Atlantic bottlenose porpoise could be blindfolded with soft latex suction cups. This method has allowed some analysis of the echo-location system of this animal, and should provide a tool for further study.

2. The blindfolded animal was remarkably adept at obstacle avoidance and in the location of small food fragments.

3. The blindfolded porpoise proved able to discriminate reward fragments from water-filled capsules of the same size, weighted to sink at the same rate as the fish. She was also able to locate and pick up fish fragments drifted within an inch of obstacles such as a hydrophone or maze poles, picking the food out of the water with the utmost delicacy, and without touching the nearby obstacles.

4. During the porpoise's approach to a reward fragment the animal emitted sounds characteristic of echo-location in the species. These were trains of short pulses whose repetition rate increased semi-systematically as the animal came nearer. Pulse repetition rates varied from a low of 16/second at the beginning of a run, to about 190/second near the fish. Then a brief lull period was usually observed just before the reward was taken, when repetition rate dropped to 26–50/second. After the fish was taken the rate sometimes rose in a "brush of pulses" to as high as 416/second. Individual pulses varied in length from 1 to 1.5 milliseconds.

5. Sporadic echo-location sounds were emitted by the animal when simply cruising around her tank. These sounds were emitted even when the animal was not blindfolded, swimming in clear water.

6. Some evidence is presented that indicates the use of a beam or cone of sound emanating from the forehead region of the animal, above the level of the mouth.

7. Attempts were made to interfere with the sound path of the blindfolded animal, to determine if disorientation would result. The first attempt, that of placing acoustically opaque suction cups over the external ear openings of the animal failed to interfere, in any visible way, with the behavior of the animal. This failure is thought to be due either to inadequate blockage of sound by the cups, or to sound conduction by adjacent blubber and muscle.

The second attempt was to train the subject to wear an acoustically opaque mask over her upper jaw and forehead region. In spite of two months' intensive training the animal refused to wear the mask for more than a few seconds. Even then she only wore it long enough to back away from the operator into open water and there to shake it off. She refused completely to swim forward with it in place. This refusal is suggestive that her sound beaming apparatus was being interfered with by the mask.

LITERATURE CITED

Fraser, F. C., and P. E. Purves, 1959. Hearing in whales. *Endeavour*, **18**: 93–98.

Griffin, D. R., 1958. Listening in the Dark. Yale University Press. New Haven, Conn.; xviii + 413 pp.

Haan, F. W. Reysenbach de, 1957. Hearing in whales. *Acta Oto-laryngologica, Suppl.*, **134**: 1–114.

Heel, W. H. Dudok van, 1959. Audio-direction finding in the porpoise (*Phocaena phocaena*). *Nature*, **183**: 1063.

Kellogg, W. N., 1953. Ultrasonic hearing in the porpoise. *J. Comp. Physiol. Psych.*, **46**: 446–450.

Kellogg, W. N., 1958. Echo-ranging in the porpoise. *Science*, **128**: 982–988.

Kellogg, W. N., 1959. Size discrimination by reflected sound in a bottle-nose porpoise. *J. Comp. Physiol. Psych.*, **52**: 509–514.

Kellogg, W. N., and R. Kohler, 1952. Reactions of the porpoise to ultrasonic frequencies. *Science*, **116**: 250–252.

KELLOGG, W. N., R. KOHLER AND H. N. MORRIS, 1953. Porpoise sounds as sonar signals. *Science*, **117**: 239–243.

MCBRIDE, A. F., 1956. Evidence for echo-location by cetaceans. *Deep-Sea Research*, **3**: 153–154.

NORRIS, K. S., AND J. H. PRESCOTT, in press. Observations of Pacific cetaceans in Californian and Mexican waters. *Univ. California Publ. Zool.*

SCHEVILL, W. E., AND BARBARA LAWRENCE, 1953. Auditory response of a bottlenosed porpoise, *Tursiops truncatus*, to frequencies above 100 kc. *J. Exp. Zool.*, **124**: 147–165.

SCHEVILL, W. E., AND BARBARA LAWRENCE, 1956. Food-finding by a captive porpoise (*Tursiops truncatus*). *Breviora, Mus. Comp. Zool., Harvard*, **53**: 1–15.

VINCENT, F., 1960. Études préliminaires de certaines émissions acoustiques de Delphinus delphis L. en captivité. *Bull. Inst. Oceanogr. Monaco*, **1172**: 1–23.

WOOD, F. G., JR., 1954. Underwater sound production and concurrent behavior of captive porpoises, *Tursiops truncatus* and *Stenella plagiodon*. *Bull. Mar. Sci. Gulf and Caribbean*, **3**: 120–133.

SKELETON FORMATION OF SEA URCHIN LARVAE. III. SIMILARITY OF EFFECT OF LOW CALCIUM AND HIGH MAGNESIUM ON SPICULE FORMATION

KAYO OKAZAKI

Department of Biology, Tokyo Metropolitan University, Setagaya-ku, Tokyo, Japan

It has been known for some time that a deficiency of calcium, magnesium or sulfate ions arrests spicule formation in sea urchin larvae (Pouchet and Chabry, 1889; Herbst, 1897, 1904). On the other hand, to the writer's knowledge, the effects of an excess of these ions have hardly been reported except with respect to calcium (Okazaki, 1956, 1960; Bevelander and Nakahara, 1960). For this reason, exposure to an excess of magnesium was tested and found to cause the same abnormality of the spicules as that previously shown to result from a deficiency of calcium ions (Okazaki, 1956).

I. Effects of Serial Changes of Calcium or Magnesium Concentration

Early developmental stages of *Hemicentrotus pulcherrimus* were used as the experimental material. Low-calcium media were prepared by mixing calcium-free sea water (containing the normal amount of magnesium) and natural sea water in

Table I

Volume ratios of stock solutions for preparing high magnesium media (2 Mg, 4 Mg)

Natural S. W.	Stock solutions					Resultant media
	M/3 $MgCl_2$	*M*/3 $CaCl_2$	*M*/2 KCl	*M*/3 Na_2SO_4	*M*/2 $NaHCO_3$	
762.0	208.0	6.5	4.3	18.2	1.0	2 Mg
285.0	618.0	21.0	14.0	58.0	4.0	4 Mg

6:4, 8:2, 9:1 and 19:1 ratios. As high-magnesium media, solutions of twice and four times the magnesium concentration of natural sea water (both with the normal amount of calcium) were prepared in the way shown in Table I, and these solutions were mixed in 3:1 and 1:1 ratios. The resultant media will be referred to as 0.4 Ca, 0.2 Ca, 0.1 Ca, 0.05 Ca, 2 Mg, 2.5 Mg, 3 Mg and 4 Mg; these values refer to the concentration of calcium or magnesium relative to that of natural sea water.

Transfer of the larvae into the experimental media was done at either of two stages: early gastrula stage with calcareous granules, or mid-gastrula stage with small triradiate spicules. The subsequent formation of the spicules in 2 Mg, 2.5 Mg, 3 Mg and 4 Mg corresponded respectively to that in 0.4 Ca, 0.2 Ca, 0.1 Ca or 0.05 Ca. The characteristics of the spicular shape in each pair of calcium-low and magnesium-high media were as follows:

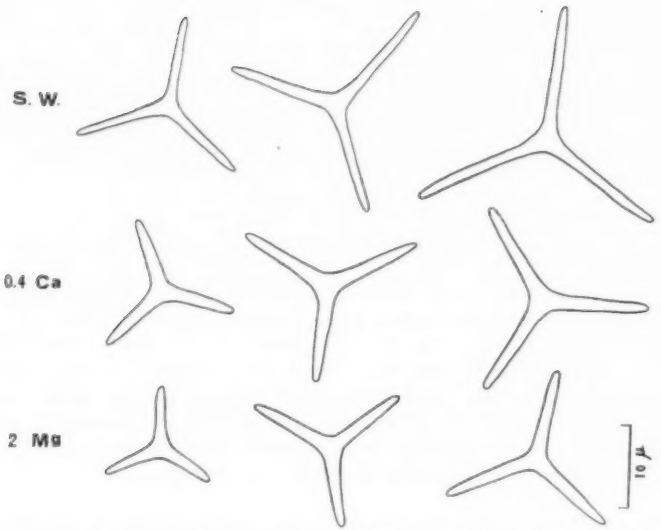


FIGURE 1. Spicules of *Hemicentrotus pulcherrimus* larvae, transferred to 0.4 Ca and 2 Mg at early gastrula stage with calcareous granules, sketched 7 hours later (13° C.).

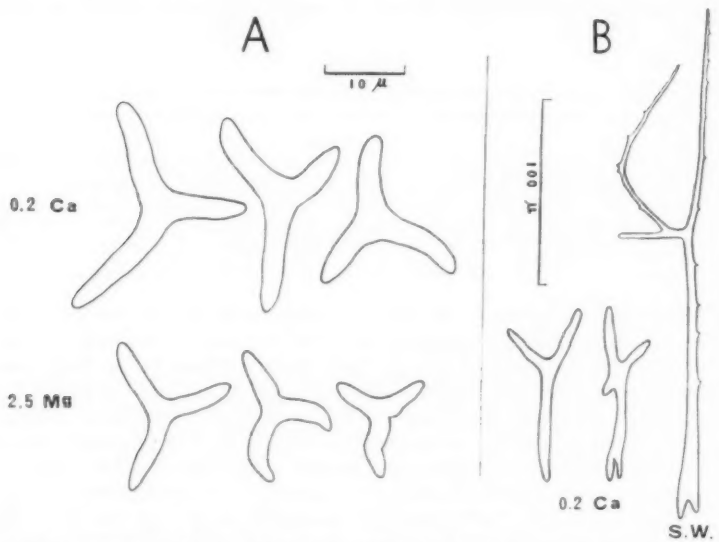


FIGURE 2. Spicules of *H. pulcherrimus* developed from calcareous granules in 0.2 Ca and 2.5 Mg. A, sketched 22 hours after transfer at 13° C. (early pluteus stage of controls); B, after 50 hours.

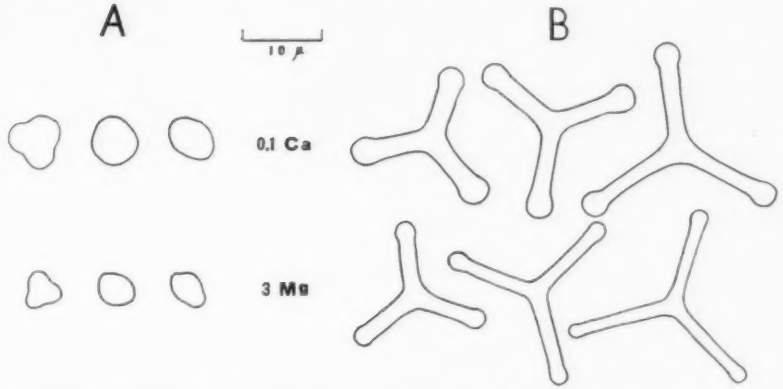


FIGURE 3. Spicules of *H. pulcherrimus* in 0.1 Ca and 3 Mg. A, transferred at calcareous granule stage and sketched 25 hours later (13° C.); B, transferred at triradiate spicule stage and sketched 23 hours later at 13° C. (compare with Figure 1).

(1) *0.4 Ca and 2 Mg.* The calcareous granules of the early gastrula acquire a triradiate form, although lengthening of the spicule is more or less retarded while its thickening is accelerated (Fig. 1). These triradiate spicules ultimately develop so that they have all the component rods of the pluteus skeleton (Fig. 2, B), which are seemingly normal but shorter and thicker than those of the sea water control. This is true even if the treatment is begun after the spicule has taken a triradiate form in sea water. On the whole, spicular growth in 0.4 Ca is superior to that in 2 Mg, but the ratios, width/length of the spicule, are much the same in these two media.

(2) *0.2 Ca and 2.5 Mg.* In comparison with the spicules found in 0.2 Ca, those in 2.5 Mg are smaller in size but quite similar in shape. Since lengthening of the spicule is strikingly inhibited, while its thickening is not so much impeded,

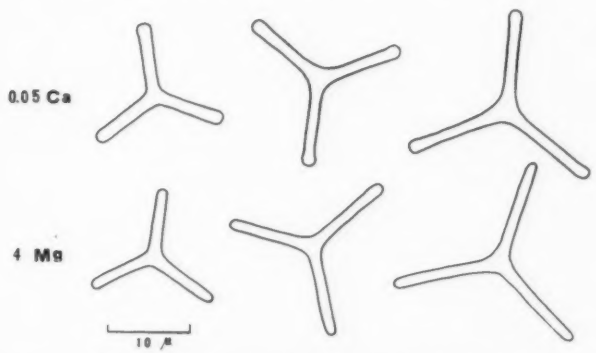


FIGURE 4. Spicules of *H. pulcherrimus* thickened in 0.05 Ca and 4 Mg. Twenty-five hours after transfer (13° C.).

the spicules become stockier than those in the previous pair of solutions. Although the calcareous granules eventually acquire three arms by the time the controls reach the early pluteus stage, they are somewhat abnormal in that the lengths of the arms and the angles between them are not uniform (Fig. 2, A). On the next day, the spicules in these media only grow to trifurcate skeletal rods when the controls develop to four-armed plutei (Fig. 2, B).

(3) *0.1 Ca and 3 Mg.* Spicules only thicken without elongating, the thickening being faster in 0.1 Ca than in 3 Mg. In these media, calcareous granules increase their masses only as spherical bodies (Fig. 3, A), and triradiate spicules, if transferred, form a spherical mass at each tip of their arms (Fig. 3, B).

(4) *0.05 Ca and 4 Mg.* Spicules thicken without increasing in length, in the same manner as in 0.1 Ca or 3 Mg, although the rates of thickening are slower (compare Fig. 3, B and Fig. 4).

II. Which is Effective, Concentration (of Calcium or Magnesium) or Ratio (Calcium/Magnesium)?

In the calcium series, the calcium concentration was reduced while the magnesium level was kept constant; in the magnesium series, the magnesium concentration was increased while the calcium level was kept fixed. This produced a continuous reduction in the calcium/magnesium ratio through the two series, leaving room for the suspicion that the similarity in the effect of low calcium and high magnesium may be due rather to a decrease in the ratio between the concentrations of the two ions than to the change of concentration, *per se*, of the respective ions. To clarify this point, the following four series of experiments were run, in which the values of calcium/magnesium were varied by mixing calcium-magnesium-free sea water, *M*/3 $MgCl_2$, *M*/3 $CaCl_2$, *M*/2 KCl and *M*/3 Na_2SO_4 in appropriate ratios. The concentrations of Mg^{++}, Ca^{++}, K^+ and SO_4^{--}

Table II

Ca and Mg levels of the experimental media

Series 1	Ca	10/10	4/10	2/10	1/10
	Mg	10/10			
Series 2	Ca	10/10	4/10	2/10	1/10
	Mg	5/10			
Series 3	Ca	10/10	4/10	2/10	1/10
	Mg	1/10			
Series 4	Ca	10/10	4/10	2/10	1/10
	Mg	10/10	4/10	2/10	1/10

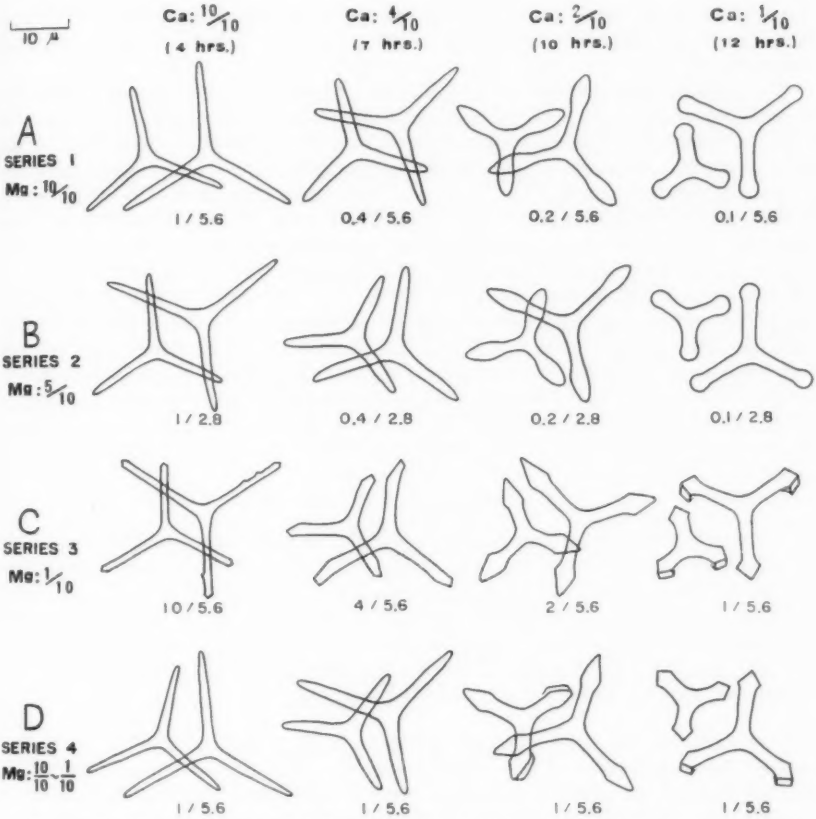


FIGURE 5. Correlation of width/length of the spicule with calcium concentration (regardless of calcium/magnesium). Calcium and magnesium concentrations expressed by fractions of normal amounts (*cf.* Table II). Fraction at bottom of each figure indicates calcium/magnesium in medium. Numerals in parentheses indicate hours after transfer.

ions were calculated on the basis that respective amounts in natural sea water are 5.6/100, 1/100, 1/100 and 2.9/100 *M*.

In each series, the concentration of calcium was changed to 10/10, 4/10, 2/10 and 1/10 that of natural sea water, potassium and sulfate being kept at the normal level. The concentration of magnesium, on the other hand, was varied among the four series; it was maintained at the normal amount in Series 1, reduced to 1/2 in Series 2 and to 1/10 in Series 3, while in Series 4, the magnesium was added in a gradient (10/10, 4/10, 2/10 and 1/10) similar to that of the calcium concentration (see Table II). The pH of each resultant medium was adjusted to 8.2 by bicarbonate buffer. Series 1 is the same as the calcium-low media used in Part 1, the left member of the series containing 10/10 calcium and 10/10 magnesium, which correspond to natural sea water in composition.

Gastrulae with small triradiate spicules were transferred to these media and after appropriate exposure, the shapes of the spicules in Series 2–4 were compared with those of Series 1.

The results are summarized in Figure 5. No difference was discernible between the spicules in Series 2 and those of Series 1 (compare Fig. 5, B with 5, A), although the calcium/magnesium ratio of the former is twice that of the latter medium. In series 3, the calcium/magnesium values were increased above the normal level, in contrast to Series 1. Notwithstanding this, the width/length of the spicule increased with a decrease in the calcium concentration to the same degree as in Series 1, although the spicular tips became square as the result of the specific influence of magnesium deficiency (compare Fig. 5, C with 5, A), the details of which will be reported in a later paper. In Series 4, the calcium/magnesium value remains the same as that of sea water. However, in 0.4 Ca-Mg (in which the calcium and magnesium concentrations were simultaneously reduced to 4/10 the normal level), the spicules were the same in all respects as those in 0.4 Ca. In 0.2 and 0.1 Ca-Mg, although the characteristic effect of magnesium deficiency was evident at the tips of the spicules as in Series 3, spicular width/length was much the same as in the corresponding medium of Series 1 (compare Fig. 5, D with 5, A).

Conclusion

These observations indicate that (a) calcium deficiency and magnesium excess in the culture medium have the same effects on spicular form, and (b) the effects are due to the absolute values of the concentrations of these ions and not to the ratios between them. This result may find an easy explanation on the basis that the magnesium ions must be competing with the calcium ions and replacing them, although they are not adequate substitutes in performing the physiological function of calcium (*cf.* Heilbrunn, 1952, pp. 538, 598).

The writer wishes to express her gratitude to Prof. K. Dan and Dr. J. C. Dan for their kind criticism and advice for the work. Her thanks are also due to the staff of the Misaki Marine Biological Station for use of the Station's research facilities.

Literature Cited

Bevelander, C., and H. Nakahara, 1960. Development of the skeleton of the sand dollar (*Echinarachnius parma*). Calcification in Biological Systems, Amer. Assoc. Adv. Sci. Washington, D. C., pp. 41–56.

Heilbrunn, L. V., 1952. An Outline of General Physiology. W. B. Saunders Co., Philadelphia, Pennsylvania.

Herbst, C., 1897. Über die zur Entwickelung der Seeigellarven nothwendigen anorganischen Stoffe, ihre Rolle und ihre Vertretbarkeit. I. Die zur Entwickelung nothwendigen anorganischen Stoffe. *Arch. f. Entw.*, **5**: 649–793.

Herbst, C., 1904. Über die zur Entwickelung der Seeigellarven nothwendigen anorganischen Stoffe, ihre Rolle und ihre Vertretbarkeit. III. Die Rolle der nothwendigen anorganischen Stoffe. *Arch. f. Entw.*, **17**: 306–520.

Okazaki, K., 1956. Skeleton formation of sea urchin larvae. I. Effect of Ca concentration of the medium. *Biol. Bull.*, **110**: 320–333.

Okazaki, K., 1960. Skeleton formation of sea urchin larvae. II. Organic matrix of the spicule. *Embryologia*, **5**: 283–320.

Pouchet, C., and L. Chabry, 1889. Sur le développement des larves d'oursin dans l'eau de mer privée de chaux. *C. R. Soc. Biol.*, **41**: 17–20.

THE UPTAKE AND TURNOVER OF S^{35} SULFATE BY LEBISTES

HAROLD L. ROSENTHAL

Dept. of Physiological Chemistry, Washington University School of Dentistry, St. Louis, Mo.

The uptake of calcium-45 and strontium-90 from the water in which they swim is linear with time for fresh-water fishes (Rosenthal, 1956, 1957), and the linear relationship is valid for the carcass, muscle, viscera and spine. The linear uptake also appears to be valid for the uptake of P^{32} phosphate and Cl^{36} chloride by brown trout (Phillips *et al.*, 1958), and for Cr^{51} chromium ion in brook trout (Knoll and Fromm, 1960). However, the rate of incorporation of calcium-45 and strontium-90 appears to be a function of the concentration of the nuclide in the water, which is adequately described by a function of the form $Y = aX^b$ where Y and X refer to rate of incorporation (Y) and water concentration (X), respectively, and a and b are constants. This relationship has recently been confirmed for strontium ion in the euryhaline *Tilapia* by Townsley *et al.* (1958–59).

The alkaline earth elements are bone-seeking elements and once incorporated into bone may remain for many days. Sulfur metabolism is also intimately associated with bone growth and development (Dziewiatkowski, 1953) but sulfate is not considered a "bone-seeker." In conjunction with other studies concerning the metabolism of mineral elements in various fishes, it was of interest to study the sulfate anion in the same manner as previously reported for calcium-45 and strontium-90 in *Lebistes* (Rosenthal, 1956–60).

Materials and Methods

Adult male wild type guppies, averaging 100 mg. in weight, were obtained from commercial sources. All fish were fed daily and maintained with commercial dry food supplemented with frozen adult brine shrimp twice weekly. Ten to fifteen fish were placed in 500 ml. artificial pond water in wide-mouth glass bowls containing carrier-free S^{35} sulfate obtained from the Oak Ridge National Laboratories. The basic solution (Prosser *et al.*, 1945), adjusted to pH 7.0, contained 0.5 mM/liter each of the following analytical reagent grade salts: sodium chloride, potassium nitrate, and magnesium chloride, and 0.2 mM/liter calcium chloride. For equilibration studies, 50–80 adult male *Lebistes* were placed in 4 liters of pond water contained in plastic bags with a surface area of 400 square inches for 7 days before the experiments were started. Additions of varying amounts of sodium sulfate were made in place of sodium chloride whenever possible.

Turnover studies were performed by placing 50–80 fish in 1500 ml. aged tap water containing S^{35} sulfate in plastic bags with a surface area of 400 square inches. After 7 days' uptake, the fish were transferred to plastic bags containing 4 liters of non-radioactive aged tap water. The fish were transferred to new water daily for the first three days and every third day thereafter until completion of the

experiment. The local tap water averaged 110 ppm sulfate (range 84–167 ppm) during the period of this study.

Samples of the carcass and tissues were dissolved in nitric acid as previously described (Rosenthal, 1956), and 0.1-ml. aliquot portions were plated on lens paper circles in stainless steel planchets. After drying under a heat lamp the samples were assayed for radioactivity in a windowless gas flow counter to a probable error of less than 5%. Under these conditions, self absorption of the sample and lens paper was a constant and all samples were corrected for self absorption and decay. The efficiency of the counter for S^{35} sulfate was 3.2×10^9 cpm/millicurie. The contribution of the isotope to the total sulfate concentration was negligible and was not considered in the calculations.

Results

As shown in Figure 1, male *Lebistes* placed in artificial pond water containing 20 ppm sulfate take up radioactive sulfate in a linear fashion during a ten-day experimental period, and the linear relationship is not affected by changes in the specific activity of S^{35} sulfate present in the water. However, the relationship between the rate of uptake of S^{35} sulfate and the activity of the water in which the fish swim at a given non-radioactive sulfate concentration is adequately de-

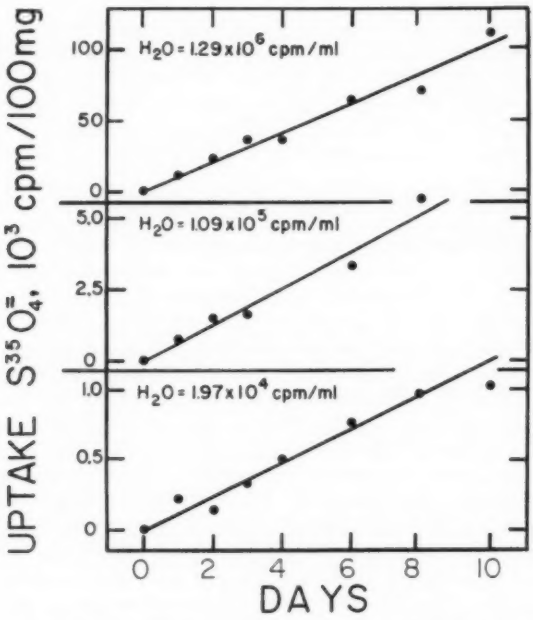


FIGURE 1. The uptake of S^{35} sulfate versus days in artificial pond water containing 20 ppm non-radioactive sulfate. Each point represents average data obtained for 4 to 8 fish.

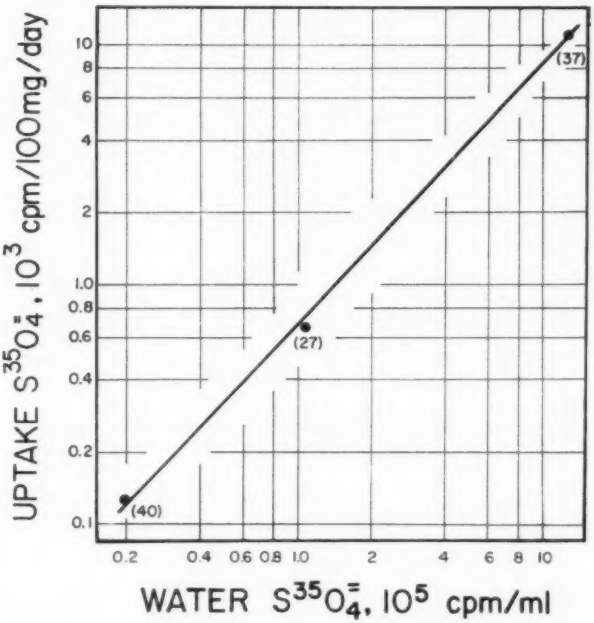


FIGURE 2. The rate of uptake of S^{35} sulfate versus the S^{35} activity of artificial pond water containing 20 ppm non-radioactive sulfate. Each point represents average data obtained for 27 to 40 animals.

scribed by a logarithmic equation of the form $Y = aX^b$, as shown in Figure 2. The rate of uptake of S^{35} sulfate is markedly affected by the non-radioactive sulfate concentration of the water (Fig. 3A). In this experiment, the uptake of S^{35} sulfate from water containing 1 ppm non-radioactive sulfate was linear for about 6 days. Between 6 to 10 days, equilibrium was established between the body of the fish and the external environment, and no further S^{35} sulfate accumulated. At concentrations between 48 ppm and 192 ppm non-radioactive sulfate, the rate of uptake of S^{35} sulfate progressively decreased as the sulfate concentration increased, and the uptake deviated from linearity after 2 to 4 days. These data indicate that the animals were essentially saturated with sulfate and the uptake of S^{35} sulfate rapidly approached equilibrium with the body sulfate pool. This interpretation was substantiated when the data were found to be adequately described by a logarithmic function (Fig. 3B).

In other experiments, the uptake of S^{35} sulfate from water of varying non-radioactive sulfate concentrations was also determined. In these experiments the fish were sacrificed 5 days after being placed in the radioactive water. It is apparent that maximal accumulation occurs when S^{35} sulfate is taken up from water containing from 1 to 20 ppm sulfate (Fig. 4). At sulfate concentrations between 20 and 192 ppm, the uptake of S^{35} sulfate decreases rapidly as the sulfate concen-

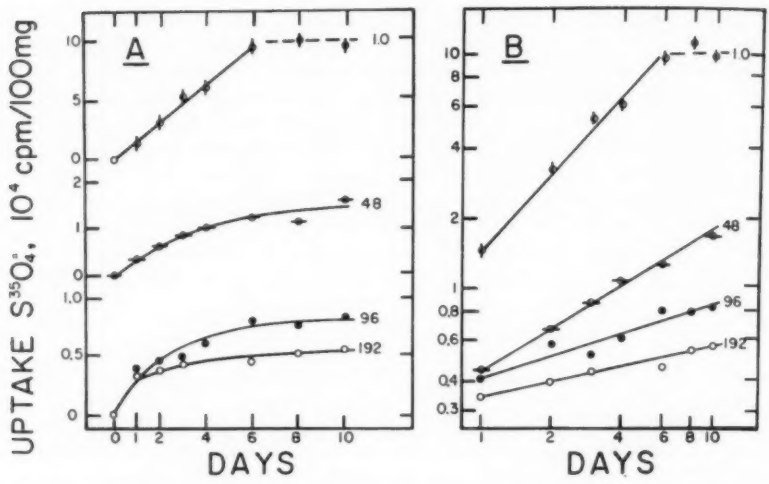


FIGURE 3. The uptake of S^{35} sulfate versus days in artificial pond water containing 3.2×10^6 cpm/ml. S^{35} sulfate. Each point represents average data obtained for 4 to 6 fish. A. Data plotted arithmetically. B. Data plotted logarithmically.

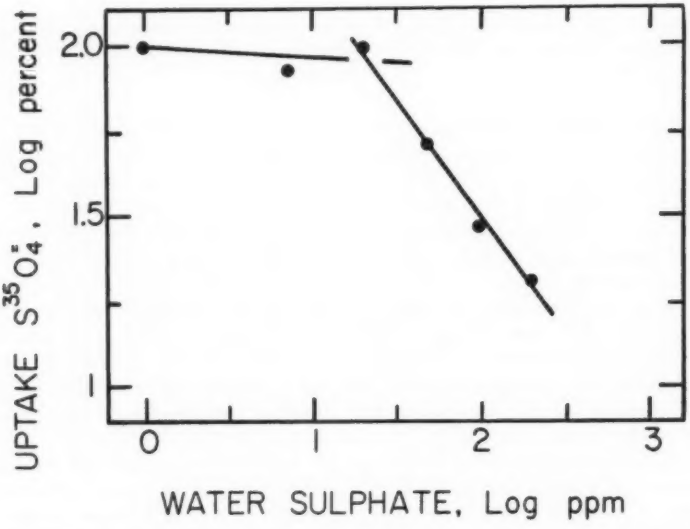


FIGURE 4. The rate of uptake of S^{35} sulfate versus concentration of non-radioactive sulfate plotted as the logarithms. Each point represents average values obtained for 3 to 6 animals. The water contained 3.7×10^5 cpm/ml.

tration increases, and the accumulation of S^{35} sulfate is logarithmically related to the sulfate concentration of the water.

That the rate of uptake of S^{35} sulfate from the water in which the fish swim is markedly affected by the body sulfate pool of the animal is shown in Figure 5. Male *Lebistes* were equilibrated for 7 days in artificial pond water containing 15 ppm sulfate. After this period of sulfate depletion, the fish were divided into three groups and transferred to bowls containing artificial pond water prepared to contain 15 ppm, 75 ppm and 200 ppm non-radioactive sulfate in addition to S^{35} sulfate. Other experiments were performed in which the fish were equilibrated in artificial pond water containing 75 ppm and 200 ppm non-radioactive sulfate before the animals were subjected to the experimental conditions.

The uptake of S^{35} sulfate by *Lebistes* after equilibration at 15 ppm appears to be non-linear when the isotope is taken up from water containing 15 ppm non-radioactive sulfate ion. The data are adequately expressed by an exponential

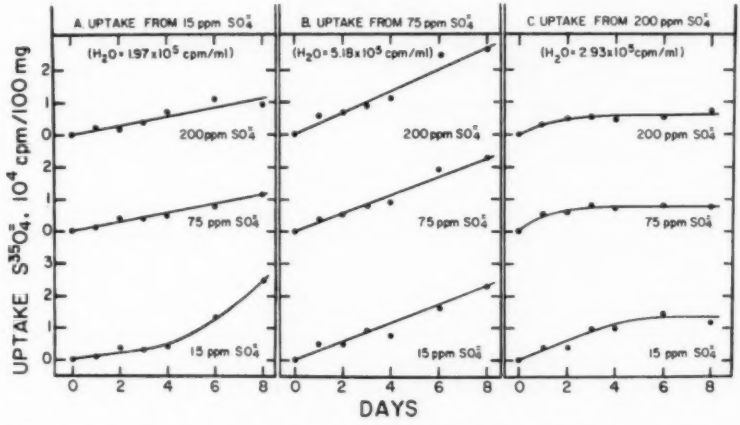


FIGURE 5. The uptake of S^{35} sulfate versus days in artificial pond water at various non-radioactive sulfate concentrations. The sulfate concentration for equilibration is given below each curve. Each point represents average values obtained for 4 to 7 animals.

equation of the form $Y = ae^{kX}$, thus indicating altered sulfate metabolism in these animals. However, the sulfate-deficient animals take up S^{35} sulfate linearly from water containing 75 ppm and 200 ppm non-radioactive sulfate.

Following equilibration at 200 ppm non-radioactive sulfate, the uptake of S^{35} sulfate reaches maximum incorporation within 2 to 3 days. This finding indicates that the sulfate stores of the animals were fully saturated and the body sulfate pool rapidly approached equilibrium with the external medium. Under all other experimental conditions, the uptake of S^{35} sulfate from the water in which the fish swim was linear during the experimental period.

The incorporation of S^{35} taken up from the water in which the fish swim is linear during a six-day experimental period for all tissues of the body that were studied (Fig. 6). The concentration of radioactivity is greatest in the soft tissues

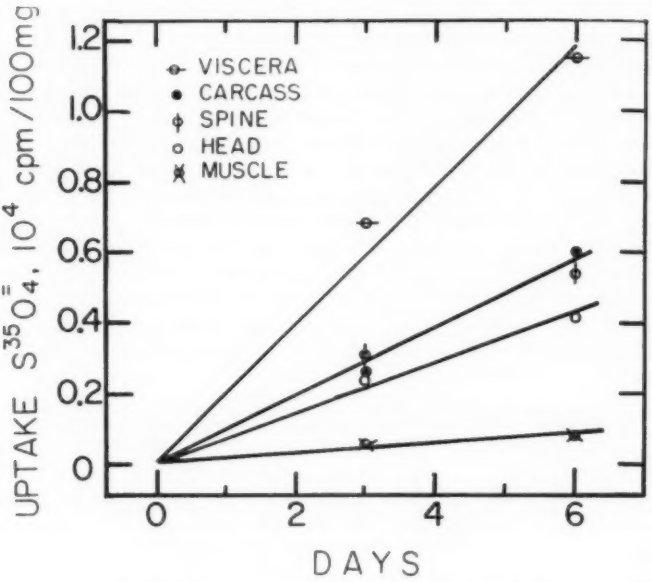


FIGURE 6. The incorporation of S^{35} sulfate into carcass and tissues versus days in artificial pond water containing 75 ppm non-radioactive sulfate. Each point represents average data obtained for 3 to 8 fish. The water contained 2.2×10^5 cpm/ml.

of the visceral organs (including the tissues of the gastrointestinal tract) while muscle incorporates the least amount and bony tissues, such as spine and head, incorporate intermediate amounts (Table I). When the data are calculated as the per cent of the total body radioactivity in the organs that were studied (Table I), 50% of the total body activity could be accounted for. The remaining 50% must have been present in the skin, scales and fins which constitute the unanalyzed remainder.

TABLE I

*Tissue distribution of S^{35} taken up from water*****

Organ	$\left(\frac{\text{Organ weight}}{\text{Body weight}}\right) \times 100$	$\left(\frac{\text{cpm/organ}}{\text{cpm/total body}}\right) \times 100$	$\left(\frac{\text{cpm/100 mg. tissue}}{\text{cpm/100 mg. body}}\right)$
Body	100	100	1.00
Head	20.25 ± 0.35	13.28 ± 1.49	0.66 ± 0.07
Viscera	13.12 ± 0.35	27.89 ± 3.22	2.44 ± 0.38
Spine	2.57 ± 0.08	2.35 ± 0.36	0.91 ± 0.12
Muscle*	40.00	6.28 ± 0.30	0.16 ± 0.02
Remainder**	24.06 ± 0.54	50.20 ± 4.60	2.10 ± 0.19

* Muscle tissue is assumed to represent 40 per cent of the wet weight of the body.
** Calculated by difference.
*** Each value represents average data obtained for 25–28 fish ± standard error of the mean.

The rate of turnover of S^{35} sulfate incorporated into body tissues was determined in fish during a 35-day experimental period. The loss of radioactivity from the body could be resolved into three well defined components (Fig. 7) with biological half-lives of 1.4 days for the most rapidly disappearing component, 10.3 days for the second component and at least 600 days for the third component. The various tissues lost radioactivity with varying rates which ranged from very rapid for viscera to very slow for muscle. The disappearance curve for muscle

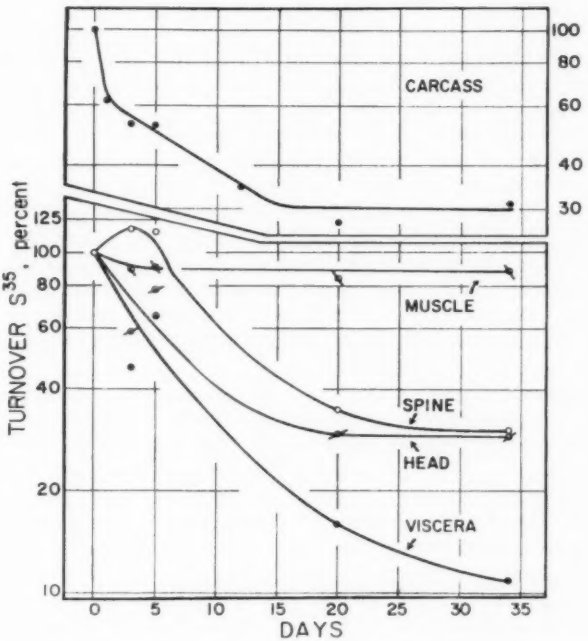


FIGURE 7. The disappearance of incorporated S^{35} sulfate from the carcass and tissues of *Lebistes* versus time in non-radioactive water. The fish initially contained 1.0×10^4 cpm/100 mg. body wt. Each point represents average data for 4 to 7 fish.

was logarithmic during the entire experimental period, and was found to have a biological half-life of 660 days.

DISCUSSION

It is apparent from these data that male *Lebistes* accumulate S^{35} sulfate from the water in which they swim in a linear fashion under specific conditions of sulfate ion concentration of the external environment. When the animals are depleted of sulfate, the rate of uptake departs from linearity when the isotope is taken up from water containing low (15 ppm) sulfate concentration. In animals saturated

with sulfate by equilibration in water containing 200 ppm sulfate, the uptake of S^{35} sulfate rapidly approaches an equilibrium. The uptake of S^{35} sulfate depends, therefore, on the physiological state of the animal and presumably on many other factors that require further study. In general, the optimum water sulfate concentration for these animals appears to be somewhat greater than 75 ppm, under the ionic and osmolar conditions of the artificial pond water used in these studies in order to maintain the sulfate integrity of the animal and to supply sufficient sulfate ion for growth and development.

The rate of uptake of S^{35} sulfate into the total carcass of *Lebistes*, and the logarithmic relationship between the rate of uptake of the isotope and the isotope concentration of the water, are very similar to that found for calcium-45 and strontium-90 in these fish (Rosenthal, 1957). Since fresh-water fishes are not considered to actively drink water (Knoll and Fromm, 1960; Reid *et al.*, 1959), the gastrointestinal tract probably plays only a minor role for the absorption of the ions. This leaves only the gills and other epithelial integuments (skin, etc.) as the regulatory tissues, although ion transport through the skin may vary with the species. In *Tilapia*, epithelial membranes seem to be permeable to calcium and strontium (Reid *et al.*, 1959) but tuna skin is not permeable to these ions (Chipman, 1956). Independent of the specific tissue involved in the accumulation of inorganic ions in *Lebistes*, the regulatory system transporting anions and cations from the water into the body and tissues of the animal appears to be the same. This conclusion was also reached for brown trout by Phillips *et al.* (1958) and for *Tilapia* by Townsley *et al.* (1958–59).

The accumulation of S^{35} in the various organs and tissues of the body may be considered to fall into three main categories. The greatest accumulation and the fastest turnover time occur in the viscera. The body, spine and head (which also includes the gills) fall into an intermediate group while muscle represents the third group with lowest concentration of S^{35} and the longest half-life. The very slow turnover rate of S^{35} in muscle ($T^{\frac{1}{2}} = 660$ days) is similar to that previously found for strontium-90 (Rosenthal, 1957) but is longer than that found for calcium-45 (Rosenthal, 1956).

A comparison of the disappearance curves for the total body and the various organs and tissues indicates that the two most rapidly disappearing components represent a loss of radioactivity from the combined viscera, spine, head and unanalyzed "remainder" while the slowest disappearance curve most probably represents the disappearance of radioactivity primarily from muscle tissue and to a lesser extent from the tissues of the spine and head. The rapid initial loss of radioactivity by the spine and head during the first 20 days indicates little, if any, sequestration of S^{35} into mineral components of these tissues. Although the turnover rate of S^{35} sulfate by tissues of the head and spine can not be adequately determined in a short term experiment of 34 days, examination of Figure 7 indicates a biological half-life ranging between 400-600 days, as determined during the last 15 days of the experiment. This value is an estimate at best and requires further study for more definitive values. Since sulfate ion is rapidly incorporated into sulfated polysaccharides of the ground substance in rats (Dziewiatkowski, 1952), the similarity of the biological half-lives for the body, spine, and muscle may represent the turnover time of this substance.

This study was performed under Contract No. AT(11-1)-678 from the U. S. Atomic Energy Commission. I am indebted to Paul R. Myers for technical assistance.

Conclusions

1. The uptake of S^{35} sulfate from the water in which the fish swim is linear with time for male *Lebistes* under specific conditions of water composition, but the uptake is modified in fishes depleted or saturated with sulfate. The rate of uptake of S^{35} sulfate is logarithmically related to the activity of the isotope in water. These results are similar to that found for calcium-45 and strontium-90, and suggest that the ion transport regulatory system is similar for all ions studied.

2. The turnover time of S^{35} sulfate for most tissues approximates a biological half-life of 600 days and presumably represents the turnover time of chondroitin sulfate and other sulfated polysaccharides.

Literature Cited

Chipman, W. A., 1956. Passage of fission products through the skin of tuna. Special Scientific Rept. No. 167. Fish and Wildlife Service. U. S. Dept. of the Interior.

Dziewiatkowski, D. D., 1952. Radioautographic studies of sulfate-sulfur (S^{35}) metabolism in the articular cartilage and bone of suckling rats. *J. Exp. Med.*, **95**: 489–496.

Dziewiatkowski, D. D., 1953. Sulfate-sulfur metabolism in the rat fetus as indicated by sulfur-35. *J. Exp. Med.*, **98**: 119–128.

Knoll, J., and P. O. Fromm, 1960. Accumulation and elimination of hexavalent chromium in rainbow trout. *Physiol. Zoöl.*, **33**: 1–8.

Phillips, A. M., H. A. Podoliak, R. F. Dumas and R. W. Thoesen, 1958. The nutrition of trout. Fisheries Research Bulletin No. 22. Cortland Hatcheries Rep. No. 27. N. Y. Conservation Dept., Albany, New York.

Prosser, C. L., W. Pervinsek, J. Arnold, G. Svihla and P. Tompkins, 1945. Accumulation and distribution of radioactive strontium, barium-lanthanum fission mixture and sodium in goldfish. U. S. Atomic Energy Comm. Tech. Inform. Serv. MDDC-496.

Reid, D. F., W. T. Ego and S. J. Townsley, 1959. Ion exchange through epithelia of fresh and sea water adapted teleost studied with radioactive isotopes. *Anat. Rec.*, **134**: 628.

Rosenthal, H. L., 1956. Uptake and turnover of calcium-45 by the guppy. *Science*, **124**: 571–574.

Rosenthal, H. L., 1957a. Uptake of calcium-45 and strontium-90 from water by fresh water fishes. *Science*, **126**: 699–700.

Rosenthal, H. L., 1957b. The metabolism of strontium-90 and calcium-45 by *Lebistes*. *Biol. Bull.*, **113**: 442–450.

Rosenthal, H. L., 1960. Accumulation of strontium-90 and calcium-45 by fresh water fishes. *Proc. Soc. Exp. Biol. Med.*, **104**: 88–91.

Townsley, S. J., D. R. Reid and W. T. Ego, 1958–59. Uptake of radioisotopes and their transfer through food chains by marine organisms. U. S. Atomic Energy Comm. Progress Report. Contract No. AT(04-3)-56.

THE PRESENCE OF DECAPOD-PIGMENT-ACTIVATING SUBSTANCES IN THE CENTRAL NERVOUS SYSTEM OF REPRESENTATIVE CIRRIPEDIA[1]

MURIEL I. SANDEEN AND JOHN D. COSTLOW, JR.

Duke University, Durham, North Carolina and Duke Marine Laboratory, Beaufort, North Carolina

The comparative distribution among the arthropods of chromatophore-activating substances has been studied by several investigators and has been reviewed recently by Carlisle and Knowles (1959). Chromatophorotropins have been shown to be present in crude extracts of the nervous systems of various malacostracans, namely representatives of the Isopoda, Natantia, Reptantia and Stomatopoda. These materials are also extractable from the corpora cardiaca of the cockroach, *Periplaneta* (Brown and Meglitsch, 1940) and from the nervous system of the horseshoe crab, *Limulus* (Brown and Cunningham, 1941).

Chromatophorotropins which play a role in the normal adaptive pigmentary responses of the Crustacea (Fingerman, Sandeen and Lowe, 1959) are believed to originate in neurosecretory cells of the nervous systems. The first study of the correlation between the distribution of a particular type of neurosecretory cell in the nervous system and the chromatophorotropic activity of extracts of portions of the nervous system was done using the crab, *Sesarma* (Enami, 1951a, 1951b). This subject is also reviewed by Carlisle and Knowles (1959).

Neurosecretory cells have been shown to be present in crustaceans lower phylogenetically than the Malacostraca. Lochhead and Resner (1958) have described their occurrence in the branchipod, *Artemia*, and Barnes and Gonor (1958) have described them in the Cirripedia, including *Pollicipes polymerus*, *Chthamalus dalli*, *Balanus glandula*, *B. hesperius laevidomus*, *B. nubilis*, and *B. rostratus*. No previous attempt has been successful, however, in finding chromatophorotropic activity in nervous system extracts of barnacles.

A chromatophorotropin which disperses the black pigment of *Uca pugilator* is one of the more widely distributed principles. Abramowitz (1937) has shown it to be present in extracts of the eyestalks of the prawn, *Palaemonetes*, the shrimp, *Crago*, as well as in extracts of the eyestalks of *Uca*. The most extensive survey of its presence was made by Brown (1940). In this study differential amounts of black-pigment-dispersing activity were found in extracts of eyestalks in the Brachyura, *Uca*, *Carcinus*, *Callinectes* and *Libinia*, in the Natantia, *Crago* and *Palaemonetes* and in the anomuran, *Pagurus*. In this same study the distribution of a substance which concentrates *Palaemonetes* red pigment was described. These two materials were found to exist in various proportions in eyestalks of the

[1] This investigation was supported in part by Grant No. B 2324 from the National Institutes of Health and by Grant No. 104-194 from the Office of Naval Research.

different crustaceans that were studied. The *Uca* black-dispersing substance is one of the chromatophorotropins that was found in *Limulus* (Brown and Cunningham, 1941). Neither these authors nor Snyder-Cooper (1938) found *Palaemonetes* red-concentrating material in extracts of the nervous system of *Limulus*.

Few interspecific tests have been made for retinal-pigment-activating substances. The probability that the light-adapting hormone is a substance distinct from the chromatophorotropins is discussed by Carlisle and Knowles (1959). Both distal pigment light-adapting and dark-adapting substances are present in extracts of eyestalks and portions of the nervous system of *Palaemonetes* (Kleinholz, 1935; Brown, Hines and Fingerman, 1952; Fingerman, Lowe and Sundararaj, 1959).

Several aspects of the life-cycle and physiology of the barnacle, *Balanus improvisus*, have been studied at the Duke Marine Laboratory (Costlow and Bookhout, 1953, 1957). As a first step in learning something about the endocrinology of the Cirripedia the following studies were undertaken in the hope of finding some homologies with other crustaceans. The fiddler crab, *Uca* and the prawn, *Palaemonetes*, about which so much is known, are common members of the fauna of this region and were selected as suitable test animals for interspecific studies.

Materials and Methods

The barnacles used in these studies were collected as needed from three different ecological niches near the Duke Marine Laboratory, Beaufort, N. C. Individuals of *Balanus eburneus* were taken from the surfaces of pilings at low tide; individuals of *Chelonibia patula*, from the carapaces of blue crabs taken in crab pots. The individuals of *Lepas* sp. were collected from a piece of driftwood carried by the currents into the vicinity of the laboratory. The fiddler crabs, *Uca pugilator*, were collected at low tide behind the laboratory buildings on Piver's Island, and the common prawns, *Palaemonetes vulgaris*, were collected from among the local algae at low tide. All of the animals were used within one to three days after being brought into the laboratory.

For all of the experiments the nervous systems of the barnacles, including supraesophageal ganglia, circumesophageal connectives and thoracic ganglia, were dissected under a dissecting microscope. This dissection actually involved the removal of the rest of the barnacle from the nervous system. With practice the whole system was obtained intact in a large percentage of the trials. Extreme care was taken to insure that no tissues other than nervous system were included. Nervous systems from ten barnacles were placed in a glass dish. Most of the water was removed with filter paper and the tissue was thoroughly triturated. This material was suspended in the desired amount of filtered sea water.

Twelve to twenty-four hours before a chromatophore assay for *Uca* black-dispersing substance, both eyestalks were removed from a group of fiddler crabs. This operation causes the pigment in the black chromatophores to concentrate and the pigment in the white chromatophores to disperse. Animals in this condition respond to injections of extracts of various portions of nervous systems of the same species. Black pigment will always disperse, and with some extracts white pigment will concentrate. The behavior of the yellow and red pigments was not considered in this study.

When testing the responses of the fiddler crabs the standard dose was 0.05 cc.

Using a hypodermic syringe and a 26-gauge needle, this was injected at the base of the fourth or fifth walking leg into the ventral hemocoel. An arbitrary system of designating concentration of materials was established. In keeping with the method used by Sandeen (1950) a concentration of one would be the equivalent of one nervous system in one dose. Since the stock suspension of barnacle nervous system extract was ten systems in 1.5 cc. sea water, there would be one nervous system in three doses, and the concentration of the suspension is referred to as a concentration of ⅓.

In order to determine the presence of chromatophorotropins for activating the pigments of *Palaemonetes,* these prawns were prepared 12–24 hours before an experiment in one of two ways. To test for red-pigment-concentrating activity both eyestalks were removed from a group of prawns, using a sharp scalpel. The eyestalks were cauterized to prevent bleeding. As a result of this operation the red pigment becomes dispersed and remains in this condition, and is responsive to an extract which produces red pigment concentration. To test for red-pigment-dispersing activity only one eyestalk was removed from specimens of prawns. These animals have their red pigment concentrated when they are placed on a white background under laboratory lighting, and yet are more responsive to injections of extracts which produce red pigment dispersion than are normal animals (Brown, Webb and Sandeen, 1952).

For the prawns, a dose of 0.02 cc. of material was used. This was injected into the animals between two of the middle abdominal segments, a little to the side of the mid-dorsal line. For these assays the barnacle extracts were made using 10 nervous systems in 0.2 cc. of filtered sea water, giving an extract with a concentration of one.

In determining chromatophore responses of both fiddler crabs and prawns the chromatophore scale of Hogben and Slome (1931) was used. In this scale the number 5 designates fully dispersed pigment and 1, fully concentrated pigment. The numbers 4, 3 and 2 designate intermediate conditions. For each assay the recipients were injected at zero time and then, at appropriate time intervals thereafter, the average chromatophore stage for each animal was determined with the aid of a dissecting microscope. The chromatophores of the fiddler crab that were observed were those on the antero-ventral aspect of the largest segment of the second or third walking leg. In the case of the prawns the large red chromatophores in the hypodermis above the heart were used. All experiments were begun between the hours of 1 and 2 PM and concluded before 6 PM in order to be working within a limited range of any diurnal rhythm of sensitivity of the chromatophores (Webb, Bennett and Brown, 1954).

To determine the presence of *Palaemonetes* distal retinal-pigment-activating substances, one-eyed prawns were prepared 12–24 hours previous to an experiment as described above. The condition of the distal retinal pigments was determined in the manner described by Sandeen and Brown (1952) with the modifications introduced by Fingerman, Lowe and Sundararaj (1959). A dissecting microscope was equipped with an ocular micrometer, and a total magnification of approximately 60 was employed. Each prawn was held ventral surface down in a petri dish of sea water under the objective. Using an appropriate balance between transmitted and reflected light two measurements of the eye were taken: the

length of the translucent area resulting from partial light adaptation at the distal edge of the eye, and the total length of the retina from the edge of the cornea to the proximal edge of the dorsal black pigment spot. The first figure divided by the second figure was used as the distal pigment index. The higher the index, the more light-adapted is the eye of the prawn.

These assay animals for distal pigment activators were kept on a black background in subdued laboratory lighting for the duration of each experiment. Under these conditions the initial condition of the distal pigment is intermediate, and if the appropriate activators are present both light-adaptation and dark-adaptation can be elicited.

Since interspecific injections were being made between Crustacea separated rather widely in their phylogenetic relationships, two additional precautions were taken. All extracts of the nervous system of the barnacles were boiled and centrifuged. The supernatant fluid, free of precipitated proteins, was used. In addition, for at least one of each type of experiment, extracts of barnacle muscle were prepared in the same manner as the extracts of nervous system and these were injected into assay animals as controls. Sea water was similarly injected into assay animals as controls for each type of experiment.

Experiments and Results

Uca black-dispersing substance

For the initial experiment each of ten eyestalkless *Uca pugilator* was injected with a dose of an extract of nervous systems of *Balanus eburneus*. As a control each of 10 similar eyestalkless *Uca pugilator* was injected with a dose of an extract of muscle of *B. eburneus*. The chromatophores of each animal were staged at 15, 30, 45, 60, 90, 120, 180, and 240 minutes following the injection. The average chromatophore stage for each group of animals for each time interval was determined and these averages were used to plot Curves A and A′ of Figure 1.

By examination of Figure 1, A, it can be seen that the extract of nervous system of the barnacle caused the black pigment of all the assay animals to become maximally dispersed (stage 5) by 45 minutes following injection. The black chromatophores remained dispersed for two hours, began to concentrate before three hours and were again concentrated after four hours. The extract of barnacle muscle produced a slight reaction in three out of ten animals. The average responses of these control animals are shown in Curve A′ of Figure 1. The white chromatophores of the fiddler crabs were affected by neither of these extracts nor by any others used in this study.

For the second experiment the goose neck barnacle, *Lepas* sp., was used. An extract of nervous systems similar to that of *Balanus* was assayed on ten eyestalkless *Uca pugilator*. One dose of filtered sea water was injected into each of six similar *U. pugilator* as a control. The chromatophores were staged at 0, 15, 30, 45, 60, 90, 120 and 150 minutes and the average value for each time was determined. These average values were used to plot Curves B and B′ in Figure 1. Curve B of Figure 1 shows that the black chromatophores of all the assay animals were maximally dispersed at 30 minutes and remained so for one hour. Concentration of pigment began between 60 and 90 minutes. At the last reading, 150

minutes, the chromatophores were still partially dispersed. By extrapolation of the curve it is apparent that the total reaction lasted at least three hours. No black pigment responses were elicited by the injection of sea water into control animals. This is shown in Curve B′ on Figure 1.

It is clear from comparison of Curves A and B of Figure 1 that there is a difference in the effectiveness of these two extracts. It seemed desirable, therefore, to compare the chromatophore responses from a series of dilutions of extracts of nervous systems of different barnacles. Since *Lepas* sp. was no longer available it was decided to use a third barnacle, *Chelonibia patula*.

Ten nervous systems of *Chelonibia patula* were dissected and extracted in 1.5 cc. sea water. As explained above the concentration of this extract was designated as one third. From this extract a series of dilutions, 1/9, 1/27, 1/81, 1/243,

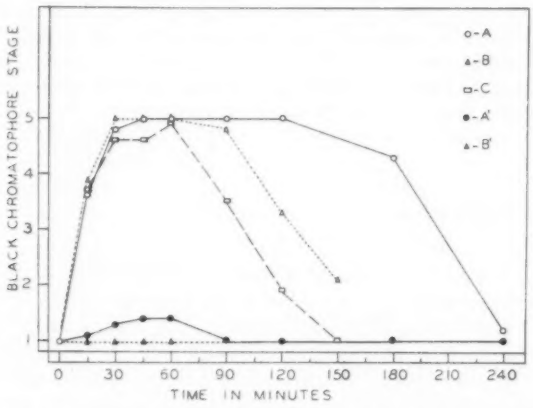


FIGURE 1. The relationships between the average black chromatophore stage of eyestalkless *Uca* and the time in minutes following the injection of extract of central nervous system of *Balanus eburneus* (A), extract of nervous system of *Lepas* sp. (B), extract of nervous system of *Chelonibia patula* (C), extract of muscle of *Balanus eburneus* (A′), sea water (B′).

were prepared. In the same manner an extract of nervous systems of *Balanus eburneus* and a similar series of dilutions were prepared. Each of these extracts was assayed on five eyestalkless *Uca*. Chromatophore stages of each individual were determined at 0, 15, 30, 45, 60 minutes and each 30 minutes thereafter until the chromatophores returned to the initial condition. The average chromatophore stage of the five animals was determined for each time that a reading was taken for each solution. From these averages a total activity value for each concentration was calculated. This was done in the following way. The number 1, designating the initial stage, was subtracted from each average value. The resultant figure represents the extent of the response noted at any given time. Then all of these values for any given concentration of extract and for the duration of the response were totaled. This activity value is, therefore, a measure of the magnitude and duration of the response. These activity values as a function of the logarithm of

dilution for both *Balanus* and *Chelonibia* are plotted in Figure 2, Curves A and B. From this figure it can be seen that a total activity of 3.6 is produced when the *Balanus* material is in a dilution of 1/243. The material from *Chelonibia*, however, produces a significant activity only up to a dilution of 1/27. Since the total activity produced by the *Balanus* extract of 1/3 concentration (24.4) is essentially the same, or even somewhat less, than that produced by the *Balanus* extract in the first experiment (25.7 for 180 minutes), it seemed wise to repeat the *Chelonibia* dilution experiment. This was done with the one modification that ten eyestalkless *Uca*, instead of five, were used as assay animals. The results were essentially the same as previously and the total activity values are plotted as a function of log of dilution as Curve C in Figure 2. It seems clear that the initial extract of nervous systems of *Chelonibia* had a concentration of active material 1/3 to 1/9 that of the *Balanus*.

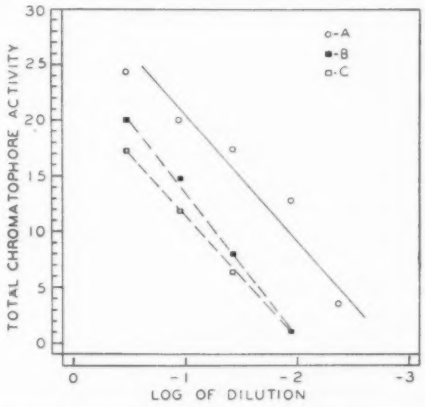


FIGURE 2. The relationships between the total chromatophore activity of the black pigment of *Uca* and the log of the dilution of extracts of nervous system of *Balanus eburneus* (A), extract of nervous system of *Chelonibia patula*, Experiment 1 (B), and extract of nervous system of *Chelonibia patula*, Experiment 2 (C).

To complete the comparison of the three genera of barnacles that were used in this study, the hourly readings of the average chromatophore stages for the nervous system extract of *Chelonibia* at 1/3 concentration from the second dilution experiment were plotted as Curve C of Figure 1. The results graphed in Figure 1 illustrate that the *Chelonibia* extract produces less chromatophore activity than the *Lepas* extract, and the *Lepas* extract produces less than the *Balanus* extract. Before any conclusion can be drawn about specific differences in this function, however, experiments would have to be designed to eliminate the possibilities (1) that there are antagonistic chromatophorotropic principles in these extracts, and (2) that the barnacles show variations as a result of their own biological activities. This is beyond the scope of the present study. It should be pointed out, however, that each barnacle extract was made initially by dissecting nervous systems from ten barnacles. This procedure was adopted in an attempt to eliminate

the possibility of individual variation among barnacles. This does not, however, eliminate the possibility of fluctuations within a whole population of animals.

In keeping with the data that are being collected on the biochemical nature of crustacean chromatophorotropins an experiment was designed to determine whether the active principle from the barnacle would be inactivated by exposure to a proteolytic enzyme. An extract of nervous systems of *Balanus eburneus* was prepared as described previously. An enzyme preparation was made using a fresh supply of Matheson Coleman and Bell trypsin powder (1–100) in a concentration of 1 part per 10,000 of filtered sea water. A portion of the enzyme was inactivated by placing it in a boiling water bath for 10 minutes. Three mixtures were prepared: (1) 3/4 barnacle CNS extract plus 1/4 boiled trypsin, (2) 3/4 barnacle CNS extract plus 1/4 active trypsin, and (3) 3/4 barnacle CNS extract plus 1/4 sea water. In tube 2, therefore, the final concentration of active enzyme was 1/40,000. These three test tubes were incubated at 40° C. for 5 minutes and then immersed in a boiling water bath for 5 minutes. The tubes were centrifuged and the supernatant taken up in syringes. Each extract was assayed on three eyestalkless *Uca*. The chromatophore stages of these assay animals were determined at 0, 15, 30, 45, 60 and 90 minutes. The extract containing boiled trypsin and that containing only the barnacle substances produced the usual black-pigment-dispersing reaction and no white pigment concentration. The extract that had been exposed to the active enzyme produced no response of the *Uca* black pigment. This experiment was repeated a second time using the shorter incubation time of one minute. The same results were obtained. The chromatophorotropic principle in the barnacle extract was completely inactivated in one minute or less by a 1/40,000 solution of trypsin.

Palaemonetes chromatophorotropic principles

To test for the presence of *Palaemonetes* red-pigment-concentrating activity three separate experiments were conducted. For each of the first two experiments, extracts of CNS of *Balanus eburneus* were prepared using ten nervous systems in 0.2 cc. sea water, and as controls, extracts of a comparable amount of muscle of *B. eburneus*. Each extract was assayed on ten eyestalkless *Palaemonetes* whose chromatophores were dispersed, with the average chromatophore stage for the groups of ten animals being 4.3 to 4.7. The chromatophores were staged at 0, 5 and 15 minutes. When the appropriate activator is present a maximal response occurs within 5 minutes. No responses of these chromatophores were observed. The two experiments were averaged together and the results are shown in Figure 3, A. To ascertain the reliability of the test a third experiment was conducted. For this experiment the activity of a similar barnacle extract was compared with that of an extract known to produce red pigment concentration, *i.e.*, 24 *Palaemonetes* eyestalks ground and suspended in 0.24 cc. of sea water. Each of these extracts was assayed on ten animals. The chromatophore stages were determined at 0, 5, 15, and 30 minutes. The average chromatophore stages for each of these times were calculated and were used to plot the curves in Figure 3, B. It can be seen from this figure that the eyestalk extract produced the expected and profound concentration of the red pigment. Again the barnacle extract showed no apparent influence on the dispersed red pigment.

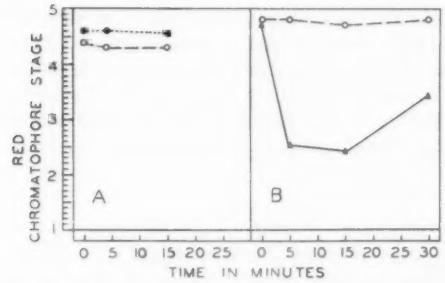


FIGURE 3. The relationship between the average stage of the red chromatophores of eyestalkless *Palaemonetes* and the time in minutes following the injection of (open circles) extract of nervous system of *Balanus eburneus;* (closed circles) extract of muscle of *Balanus eburneus;* and (open triangles) extract of eyestalks of *Palaemonetes.*

To test for the presence of *Palaemonetes* red-dispersing substance four experiments were conducted. For the first three experiments nervous system extracts of barnacles were prepared in the usual manner. Two types of control solutions were used; one was sea water and the other, an extract of supraesophageal ganglia of *Palaemonetes,* ten ganglia in 0.2 cc. of sea water. The extract of supraesophageal ganglia of *Palaemonetes* is known to produce dispersion of the red pigment of one-eyed *Palaemonetes* (Brown, Webb and Sandeen, 1952). In the fourth experiment three separate extracts of muscle of *Balanus* were used. Each of these extracts was assayed on a group of ten one-eyed *Palaemonetes.* The chromatophores were staged at 0, 15, 30, 60, 90, and 120 minutes in all except the third experiment in which the zero reading was omitted. Average values for each of the time intervals were determined for each extract.

There were, therefore, three experiments of ten animals each, for each kind of extract. Since the results were qualitatively the same for these three trials the averages for the three were calculated. These average values were used to plot the curves which are shown in Figure 4. It can be seen in Figure 4 that

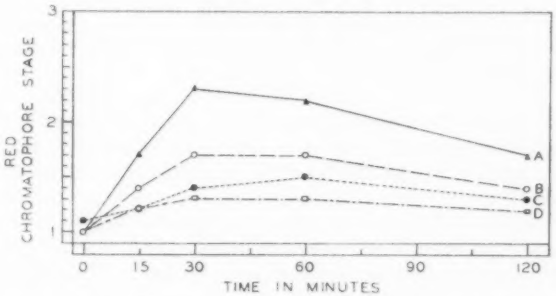


FIGURE 4. The relationship between the average stage of the red chromatophores of one-eyed *Palaemonetes* and the time in minutes following the injection of extract of supraesophageal ganglia of *Palaemonetes* (A), extract of nervous system of *Balanus* (B), extract of muscle of *Balanus* (C), sea water (D).

the extract of supraesophageal ganglia of *Palaemonetes* produced the predicted response with an average chromatophore dispersion of 2.3 at 30 minutes. Both the injection of sea water and the injection of extract of barnacle muscle produced a slight response. The extract of nervous systems of *Balanus*, however, produced a decided dispersion of the red pigment, but of less magnitude than did the extract of supraesophageal ganglia of *Palaemonetes*.

Distal retinal-pigment-activators

To test for the presence of distal retinal-pigment-activators, ten one-eyed *Palaemonetes* were placed in each of three small black-painted enamel pans for two hours before the beginning of each experiment. Before receiving an injection the distal pigment index of each animal was determined as described under

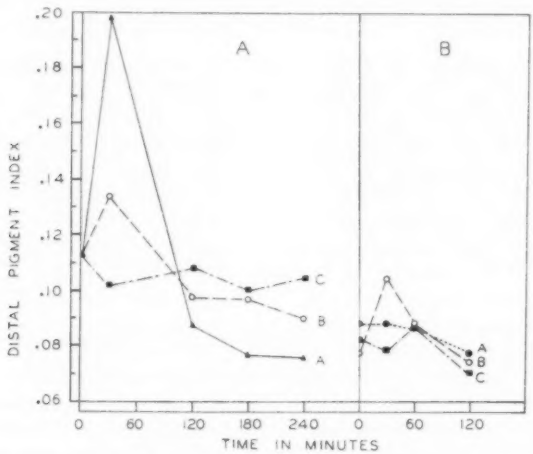


FIGURE 5. Part A. The relationship between the average distal pigment index of one-eyed *Palaemonetes* and the time in minutes following the injection of extract of eyestalks of *Palaemonetes* (A), extract of nervous system of *Balanus* (B), sea water (C). Part B, following the injection of extract of muscle of *Balanus* (A), extract of nervous system of *Balanus* (B), sea water (C).

Materials and Methods. The average value for each group of ten animals was calculated. Under these conditions these groups of animals had average distal pigment indices ranging from .083 to .116, indicating an intermediate degree of light adaptation.

In the search for distal-pigment-activators four experiments were performed. For the first two experiments extracts of the nervous systems of *Balanus eburneus* were prepared using ten systems in 0.2 cc. sea water. Extracts of eyestalk of *Palaemonetes*, 24 in 0.24 cc. sea water, were also prepared to be used to produce both light-adaptational and dark-adaptational responses, known to occur (Fingerman, Lowe and Sundararaj, 1959). Injections of sea water into individuals in the third group of animals were used as controls for each experiment.

The results of these two experiments were qualitatively the same and the average distal pigment indices for each extract were averaged together. These average values for the two experiments were used to plot the curves shown in Figure 5, A. The extract of *Palaemonetes* eyestalk produced the expected light-adaptation and dark-adaptation of the distal pigment. The extract of nervous system of barnacles produced a small amount of light-adaptation and no dark-adaptation. The sea water controls remained relatively constant for the duration of the experiment.

For the second two experiments the activities of extracts of the nervous systems of *Balanus* were compared with similar extracts made from muscles of *Balanus*. Injections of sea water were used on a third group of animals as controls for each experiment. These two experiments were qualitatively the same and average distal pigment indices for each time interval were averaged together. These values were used to plot the curves shown in Figure 5, B. In these experiments all animals became more dark-adapted as the experiment progressed. This was probably due to the fact that the laboratory became darker as the experiment continued into the later afternoon. The extract of the nervous system of barnacles again produced a small but noticeable light-adaptational response, but no dark-adaptation. The extract of muscle of barnacles produced no change in the distal pigment index.

Discussion

Until recently all of our knowledge of crustacean endocrinology has been gained from studies of major groups of the Subclass Malacostraca. Carlisle and Knowles (1959) have stated that the lower groups of Crustacea have many functions "suggestive of endocrine control." The technical problems of studying these groups of relatively small animals are, of course, serious. The contribution of the present study is the demonstration that representatives of one of these lower groups, the Subclass Cirripedia, have substances in their nervous systems homologous in action to materials in the decapods when tested on certain decapods.

The most obvious property of the extracts of the central nervous system of the barnacles was found to be that of dispersing the black pigment of eyestalkless *Uca pugilator*. In three experiments described in this paper and numerous others performed in connection with other studies, the most concentrated extracts of *Balanus eburneus* that have been used have produced black-pigment-dispersal which lasts for approximately four hours.

This same property has been found in two other barnacles, *Chelonibia patula* (two experiments) and *Lepas* sp. (one experiment). Although there is a limited amount of data it appears that these second two genera contain this material in an amount 1/3 to 1/9 that found in *Balanus eburneus*. By using nervous systems from a group of barnacles for each extract the attempt was made to eliminate the influence of individual variation among barnacles. Care was also taken to insure that the assay animals in all experiments were essentially similar physiologically. It remains a possibility, therefore, that *Balanus* and *Chelonibia*, within the same family but different subfamilies of barnacles, and *Lepas*, in a separate suborder, have characteristics as different as are found in the pigmentary physiology of *Crangon* and *Palaemonetes*, which are in different families of the Suborder Natantia.

In the dilution experiment it was shown that the black-dispersing substance of *Balanus eburneus* is active in a dilution of 1/243. The activity obtained at this concentration is comparable to that obtained with the same concentration of an extract of supraesophageal ganglia of *Uca pugilator* (Sandeen, 1950).

Even though it is believed that these materials are secreted by specialized cells which are not necessarily evenly distributed throughout the nervous system, it was thought desirable to attempt a comparison of the sizes of these tissues. To do this a Whipple disc was placed in the ocular of a dissecting microscope. Using a magnification of approximately 27 the whole nervous system of *Balanus eburneus* was drawn to scale on graph paper. Using the same scale, drawings were made of the supraesophageal ganglia and circumesophageal connectives of *Palaemonetes vulgaris* and *Uca pugilator*. These nervous systems were dissected from animals

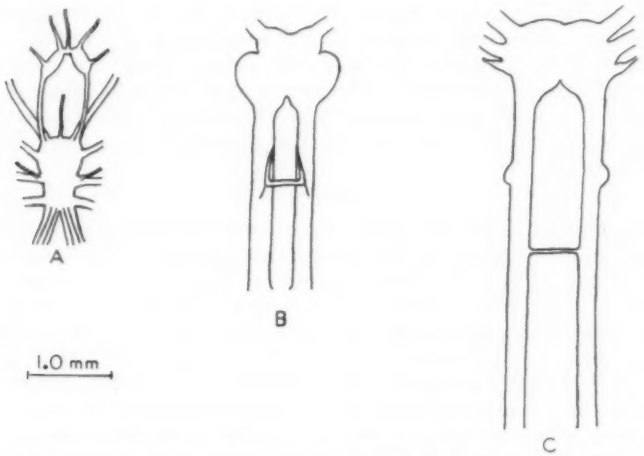


FIGURE 6. Diagrams of the central nervous system of *Balanus eburneus* (A), supraesophageal ganglia and circumesophageal connectives of *Palaemonetes vulgaris* (B), and the supraesophageal ganglia and circumesophageal connectives of *Uca pugilator* (C).

of the average size used in all of the experiments. This comparison, of course, is only partially satisfactory because it is not a measurement of volume. These three drawings are shown in Figure 6.

By examination of this figure it is apparent that the total area of the nervous system of *Balanus* is approximately three-fourths that of the supraesophageal ganglia of *Uca*. These tissues of somewhat comparable size give essentially similar black-dispersing activity. Until histological studies are undertaken, nothing can be said about the presence and comparative distribution of cells responsible for producing the active materials. This finding does suggest, however, that the tissue as dissected probably does not include any sort of storage organ equivalent to the sinus gland of the decapod, since an extract of the sinus gland will produce a significant black-pigment-dispersion at a dilution of 1/729 (Sandeen, 1950). The fact that the extract of *Balanus* produces a chromatophore response at a dilution of 1/243,

as well as the facts that this activity is not destroyed by boiling but is destroyed by trypsin, lends support to the conclusion that we are dealing with substances homologous to those found in the decapods.

In the experiment using *Palaemonetes* as an assay animal it was shown that extracts of the nervous system of *Balanus eburneus* produce a red-pigment-dispersion and no red-pigment-concentration. When these results are compared with those obtained on the relative activities of the parts of the nervous system of *Palaemonetes* (Brown, Webb and Sandeen, 1952), some interesting contrasts are apparent. In these latter experiments extracts of all parts of the nervous system, except the circumesophageal connectives, produced greater red-pigment-dispersion (chromatophore stage of approximately 2 at 30 minutes) than did the barnacle extract (1.7 at 30 minutes). Extracts of the connectives of *Palaemonetes,* on the other hand, produced a red-pigment-dispersion, similar to that of the barnacle extract, of only about 1.7. In *Palaemonetes,* it is the circumesophageal connectives which also contain the largest amount, outside of the eyestalk, of red-pigment-concentrating activity. It was a surprise, therefore, to find low dispersing activity and yet no red-concentrating activity.

For the experiments of Brown, Webb and Sandeen (1952) the extracts contained the equivalent of one-half a portion (i.e., a supraesophageal ganglion or one connective per dose of 0.025 cc.). In these experiments on barnacles the extracts contained the equivalent of a whole nervous system in one dose of 0.02 cc. In Figure 6 it can be seen that the supraesophageal ganglia of *Palaemonetes,* as dissected for these studies, are almost equivalent in size to those of *Uca.* Since a concentration of one was used in this study, as compared to the concentration of 1/2 used by Brown, Webb and Sandeen (1952), it seems clear that the amount of red-pigment-dispersing substance present is small.

One other situation exists in which Uca black-dispersing substance occurs without the co-existence of *Palaemonetes* red-concentrating material. This is the case with extracts of the nervous system of *Limulus* (Brown and Cunningham, 1941; Snyder-Cooper, 1938). These findings lend support to the view that each of these chromatophorotropins has some kind of individuality.

Finally, it has been shown that the barnacle nervous system contains some *Palaemonetes* distal retinal pigment-light-adapting substance and no dark-adapting material. This result can be compared with that obtained by Fingerman, Lowe and Sundararaj (1959). Whereas they obtained both light- and dark-adaptation of *Palaemonetes* following injection of extracts of eyestalks of *Palaemonetes,* they obtained only a light-adapting response following injection of extract of the supra-esophageal ganglia of *Palaemonetes* in a concentration of 1/3.

Barnacles are very different morphologically from the decapod crustaceans. They do not have compound eyes, nor do they have chromatophores. Pigments do occur in the mantle tissue which secretes the shell. It is possible, however, that the physiologically active substances demonstrated here do not function for the barnacle as they do for the decapod. They may serve in molting or reproduction, two functions which are hormonally regulated in the decapods. An equally likely hypothesis is that these substances which are active on the decapods co-exist with related compounds of barnacles having their own functions, and thus simply describe another systematic affinity among crustaceans. The very interesting find-

ing of Fingerman and Mobberly (1960) that blind cave crayfish with no retinal pigments and no chromatophores have both distal-pigment-light-adapting hormone and red-pigment-concentrating material causes one to consider a third possibility, that these materials in barnacles are vestiges from a primitive ancestor that employed them in a way similar to that of modern decapods.

Summary

1. A *Uca* black-pigment-dispersing substance has been extracted from the nervous systems of three genera of barnacles, *Balanus eburneus, Chelonibia patula* and *Lepas* sp. This substance is heat-stable and can be inactivated by trypsin.

2. The *Uca* black-pigment-dispersing substance from *Balanus eburneus* is active in a dilution of 1/243. This activity is comparable to that known to be produced by a similar extract of supraesophageal ganglia of *Uca pugilator*.

3. The nervous system extracts of these barnacles do not contain *Palaemonetes* red-pigment-concentrating material, but do contain a small amount of *Palaemonetes* red-pigment-dispersing material.

4. *Palaemonetes* distal retinal pigment-light-adapting principle was demonstrated to be present in small quantity but distal retinal pigment-dark-adapting principle was not shown to be present.

5. The relationship of these findings to some of our knowledge of crustacean hormones is discussed.

Literature Cited

Abramowitz, A. A., 1937. The comparative physiology of pigmentary responses in the Crustacea. *J. Exp. Zool.*, **76**: 407–422.

Barnes, H., and J. J. Gonor, 1958. Neurosecretory cells in some cirripedes. *Nature*, **181**: 194.

Brown, F. A., Jr., 1940. The crustacean sinus gland and chromatophore activation. *Physiol. Zoöl.*, **13**: 343–355.

Brown, F. A., Jr., and O. Cunningham, 1941. Upon the presence and distribution of a chromatophorotropic principle in the central nervous system of *Limulus*. *Biol. Bull.*, **81**: 80–95.

Brown, F. A., Jr., M. N. Hines and M. Fingerman, 1952. Hormonal regulation of the distal retinal pigment of *Palaemonetes*. *Biol. Bull.*, **102**: 212–225.

Brown, F. A., Jr., and A. Meglitsch, 1940. Comparison of the chromatophorotropic activity of insect corpora cardiaca with that of crustacean sinus glands. *Biol. Bull.*, **79**: 409–418.

Brown, F. A., Jr., H. M. Webb and M. I. Sandeen, 1952. The action of two hormones regulating the red chromatophores of *Palaemonetes*. *J. Exp. Zool.*, **120**: 391–420.

Carlisle, D. B., and F. Knowles, 1959. Endocrine Control in Crustaceans. Cambridge University Press.

Costlow, J. D., Jr., and C. G. Bookhout, 1953. Moulting and growth in *Balanus improvisus*. *Biol. Bull.*, **105**: 420–433.

Costlow, J. D., Jr., and C. G. Bookhout, 1957. Body growth versus shell growth in *Balanus improvisus*. *Biol. Bull.*, **113**: 224–232.

Enami, M., 1951a. The sources and activities of two chromatophorotropic hormones in crabs of the genus *Sesarma*. I. Experimental analyses. *Biol. Bull.*, **100**: 28–43.

Enami, M., 1951b. The sources and activities of two chromatophorotropic hormones in crabs of the genus *Sesarma*. II. Histology of incretory elements. *Biol. Bull.*, **101**: 241–258.

Fingerman, M., M. E. Lowe and B. I. Sundararaj, 1959. Dark-adapting and light-adapting hormones controlling the distal retinal pigment of the prawn *Palaemonetes vulgaris*. *Biol. Bul.*, **116**: 30–36.

FINGERMAN, M., AND W. C. MOBBERLY, 1960. Trophic substances in a blind cave crayfish. *Science*, **132**: 44–45.

FINGERMAN, M., M. I. SANDEEN AND M. E. LOWE, 1959. Experimental analysis of the red chromatophore system of the prawn *Palaemonetes vulgaris*. *Physiol. Zoöl.*, **32**: 128–149.

HOGBEN, L. T., AND D. SLOME, 1931. The pigmentary effector system. VI. The dual character of endocrine coordination in amphibian colour change. *Proc. Roy. Soc. London, Ser. B*, **108**: 10–53.

KLEINHOLZ, L. H., 1935. Crustacean eyestalk hormone and retinal pigment migration. *Biol. Bull.*, **70**: 159–184.

LOCHHEAD, J. H., AND R. RESNER, 1958. Functions of the eyes and neurosecretion in crustacea Anostraca. XVth International Congress of Zool., Sect. IV, Paper 21.

SANDEEN, M. I., 1950. Chromatophorotropins in the central nervous system of *Uca pugilator*, with special reference to their origins and actions. *Physiol. Zoöl.*, **23**: 337–352.

SANDEEN, M. I., AND F. A. BROWN, JR., 1952. Responses of the distal retinal pigment of *Palaemonetes* to illumination. *Physiol. Zoöl.*, **25**: 222–230.

SNYDER-COOPER, R., 1938. Probable absence of a chromatophore activator in *Limulus polyphemus*. *Biol. Bull.*, **75**: 369.

WEBB, H. M., M. F. BENNETT AND F. A. BROWN, JR., 1954. A persistent diurnal rhythm of chromatophoric responses in eyestalkless *Uca pugilator*. *Biol. Bull.*, **106**: 371–377.

A CYTOLOGICAL STUDY OF THE ORIGIN OF MELANOPHORES IN THE TELEOSTS[1]

DAVID C. SHEPHARD

Department of Zoology, University of Chicago, Chicago 37, Illinois

Conclusive evidence for the neural crest origin of all teleost melanophores does not exist despite the growing body of evidence that the neural crest does give rise to melanophores in many fish species. Extirpation, explantation, and transplantation experiments by a number of investigators on a variety of species definitely indicate that the neural region of teleost embryos is a source of future melanophores (Lopashov, 1944; Humm and Young, 1956; Kajishima, 1958). Descriptive findings, from one of the first suggestions that melanophores stem from neural ectoderm (Borcéa, 1909), to the recent extensive work of Orton (1953), lend further support to the concept that the neural crest is a source of melanophores in fish. In fact, a tabulation of the data from the literature cited in the present paper shows some sixty species of fish, representing more than twenty families and ten orders, in which there is some evidence for the neural crest origin of melanophores; however, none of this work constitutes evidence that the crest is the sole source of these cells. Conversely, Trinkaus (1951) has removed the entire embryo at very early stages, and the remainder of the egg produced a normal complement of melanophores simultaneously with their appearance in unoperated eggs. The species used in his study was *Fundulus heteroclitus*, of which Stockard (1915) has given a detailed description of the origin of melanophores from the extra-embryonic germ ring. Stockard's observations have been supported by other authors (Gilson, 1926; Russel, 1939). Further evidence that the germ ring may give rise to melanophores is contributed by the experimental studies of Oppenheimer (see 1949), although she does not agree that this structure is a normal source of melanophores. A search of the cited literature yields evidence for this secondary site of pigment cell origin in only *F. heteroclitus* and possibly *Epiplatys fasciolatus* (Oppenheimer, 1938). Even *Fundulus majalis* seems to lack these cells, judging from the description in Bancroft (1912). These exceptional pigment cells are found largely if not solely in the extra-embryonic yolk sac membranes of a very few species of cyprinodont fishes, and in *F. heteroclitus* they all eventually encircle the blood vessels of the yolk sac—a phenomenon, which is unusual and possibly unique among fish embryos (Stockard, 1915).

There are still numerous problems concerning the origin of fish melanophores, however. Fish do not have a morphological neural crest as seen in the higher vertebrates. The experiments cited above involved removal of pieces of the ectoderm plus some of the underlying neural keel, and hence are not truly analogous with removal of the neural folds in higher forms. The descriptive data are con-

[1] This investigation was carried out during the tenure of a Predoctoral Fellowship from the National Cancer Institute, United States Public Health Service.

fined to reports of the melanophores or their immediate precursors first being seen in the "neural region." Newth (1951, 1956) has studied the crest and its derivatives in the lamprey. He describes the crest as a histologically distinct region between the keel and the overlying ectoderm. Kajishima (1958) mentions very briefly that he sees a similar region in the goldfish, but he does not figure it. There appears to be no published study identifying the neural crest of teleosts and determining its extent and time of migration. Goodrich (1950) is even led to state (p. 18) that "there does not at present appear to be available critical evidence to demonstrate the origin of fish chromatophores from the neural crest. . . ."

Concerning the extra-embryonic origin referred to above there is even less unanimity. Many of the descriptions predate DuShane's (1935) demonstration of the neural crest origin of amphibian pigment cells. Oppenheimer and Trinkaus have worked on this problem more recently, and Trinkaus (1951) contends there is a normal extra-embryonic source of pigment cells in *Fundulus heteroclitus*, while Oppenheimer (1949), working on the same species, attributes her results to a "bedeutungsfremde Selbstdifferenzierung" of cells in an abnormal situation.

The object of the present investigation is to describe the neural crest of several species of teleosts and to provide some information on its time of migration. The yolk sac melanophores of *F. heteroclitus* are identified at an early stage and followed throughout their differentiation to demonstrate more conclusively that in this species, and possibly in a few others, there is normally an extra-embryonic source of melanophores.

Materials and Methods

The embryos of several species of fish were used for this study. Much of the work on the neural crest was done on the zebra fish (*Brachydanio rerio*), a tropical cyprinid which is easily raised and bred in the laboratory. Its eggs are small (0.6 mm. in diameter) and transparent enough for observation with the phase microscope. The egg is reasonably good for histological work, too, since the chorion is easier to remove than those of most fishes, and it offers less difficulty in sectioning than the larger eggs.

The eggs of *Fundulus heteroclitus*, a cyprinodont, and those of the cunner (*Tautogolabrus adspersus*), a labrid, were studied at the Marine Biological Laboratory in Woods Hole, Massachusetts. *Fundulus* has an egg which is approximately 2 mm. in diameter, large enough for some experimental work, but sufficiently transparent to allow good observation of the cells on the yolk sac in the living egg. The cunner has a smaller egg, less than 0.5 mm. in diameter, and it has been described as "glass clear." It is pelagic and too delicate for most kinds of work.

Several other tropical fish were used briefly. Their taxonomy and handling were based mostly on Axelrod and Schultz (1955). The methods used with *Fundulus* and the cunners were taken from Costello *et al.* (1957). The histological procedures in the latter reference (page 225) were used with many variations. Flemming's seems to be a more satisfactory fixative, and an isopropyl dehydration and clearing can be substituted for the ethanol-amyl acetate procedure. The stain most frequently used was Ehrlich's hematoxylin and eosin. When it was desirable to see the earliest pigment granules, safranin in 50% ethanol was used to allow visualization of the nuclei and cell outline. Basophilia was judged by eye on

sections stained in pH 4 azure-B. Sections were cut at 6 μ, or thinner when better resolution was desired. Embryos to be examined whole were fixed in Stockard's (1915) fixative.

Since the pigment and propigment cells form a rather diffuse tissue, if they may be called a tissue at all, sectioned material is not ideal for observing them. Any plane of sectioning through the embryo would give good results for only a few of these flattened and irregularly shaped cells. A procedure was developed which allowed satisfactory observation of these cells in their entirety on the *Fundulus* yolk sac. The live embryo was laid on its side on a clean coverslip. The yolk sac was punctured on its top surface, and the yolk and fluid around the embryo drawn away with filter paper leaving the embryo flattened out on the coverslip. The coverslip with the embryo on top was set on an aluminum rod, the other end of

TABLE I

Zebra fish stage sequence at 28° C.

Stage No.	Age in hours	Distinguishing characteristics (pigment cell appearance)	Hisaoka and Battle stage
15	15	Blastopore closure	17
16	16+	Optic vesicles visible	17
17	18	Formation of first somites	17
18	21	Auditory placodes formed, ten somites	18
19	23	Cavity in central nervous system	18
20	27	Partial constriction of yolk mass (First pigment granules appear)	19
21	30	Motility of embryo (Visibly pigmented eye)	20
22	33	Beginning of heart beat (First body melanophores visible)	21
23	35	Circulation begins (Melanophores appear on yolk)	22
24	39	Circulation on yolk sac (Great increase in general pigment)	22
25	45	Prominent fin buds (Some expanded melanophores on body and yolk sac)	22

which was immersed in liquid nitrogen (dry ice-acetone mixture when liquid nitrogen was not available). The embryo was thus frozen almost instantaneously. Under a dissecting microscope the top layers of the frozen embryo were carefully shaved away with a sharp scalpel or razor blade. Ideally, a layer consisting of most of one side of the embryo but only a few cell layers thick could be left on the coverslip. This tissue was then instantaneously thawed and fixed by dropping the coverslip into acetic-alcohol (1:3). The fixative precipitates the protein so that the tissue adheres to the glass. It is subsequently treated as sectioned material.

Living embryos were observed and staged with a dissecting microscope having a highest magnification of 80×. A phase contrast microscope was used extensively. It was found that the Zeiss 40× oil phase objective made it possible to observe many intracellular details in the living fish embryos. A filar ocular micrometer was used to obtain measurements on fixed material.

At the time this study was begun, there was no set of normal developmental stages for the zebra fish and cunner. Oppenheimer's (1937) stages were used for *Fundulus*. Since there is no stage sequence available for the cunner, the observations presented here are based on the age of the embryos in hours at approximately 17° C. A modification of Oppenheimer's stages was developed for the zebra fish; however, they cover only the period extending from the closure of the blastopore to the complete differentiation of the earlier pigment cells, and are based as much as possible on the pigment cells themselves. Recently the development of the zebra fish has been described (Hisaoka and Battle, 1958), but the stages given are too coarse for the purposes of the present study. Table I presents the stages used herein and a comparison of them with those of Hisaoka and Battle.

Observations

The teleost neural crest

In the zebra fish, the melanoblasts begin to migrate before they become pigmented. The first melanin that can be seen is in the eye, and it is followed by a wave of melanogenesis progressing posteriorly. Since there are slight differences in the time of appearance of melanin and since the crest also shows some modification in the head region and in the tail bud, the sequence of events to be described will be concerned mainly with the trunk region.

At stage 15, the embryonic axis of the zebra fish is represented only by a swelling in the midline of the embryonic shield with some separation of the notochord (Fig. 1). As stage 16 arrives, there is a well developed neural keel in the brain region, while in the trunk the keel is represented by a ridge in the ectoderm above a well delimited notochord (Fig. 2). Figures 3 to 8 show the trunk region in stages 17 to 24. It will be seen that the neural primordium becomes progressively larger and more discrete. Initially the dorsal surface of the keel is broadly attached to the ectoderm (Figs. 3 and 4). This attachment narrows in stage 18 (Fig. 5), and the cells constituting it become loose in stage 18 to 19 (Fig. 6). It is these cells that apparently represent the neural crest of the zebra fish embryo. In Figure 7, early migrating cells can be seen leaving the crest, and the first pigment granules are forming in some of these cells. Figure 8 shows the paths of migration outlined by pigmented cells, most of which have now ceased migration.

The majority of the melanoblasts first migrate between the neural tube and the somites and then down onto the surface of the notochord. From there some of them follow the remnants of what has been referred to as the segmentation cavity, to the yolk sac. Most of the cells that have become heavily pigmented along this route cease their migration and are eventually associated with various internal organs. Another path of migration is between the ectoderm and the somites. The cells in this position are easily visible with the phase microscope (Figs. 9 and 10), and are the ones that form the larval pigment. The paths of migration are quite similar to those described for the higher vertebrates.

Cytologically these cells are not as desirable for the study of melanoblast differentiation as those of the *Fundulus* yolk sac which will be described in a later section; however, some of the same phenomena are observable. Prior to

SYMBOLS USED IN FIGURE LEGENDS

B—Melanoblast
C—Notochord
E—Endothelial cell
N—Neural primordium
M—Melanophore
X—Neural crest

The scale marker is 10 μ long in all figures except Figures 17 and 23 where it is 100 μ.

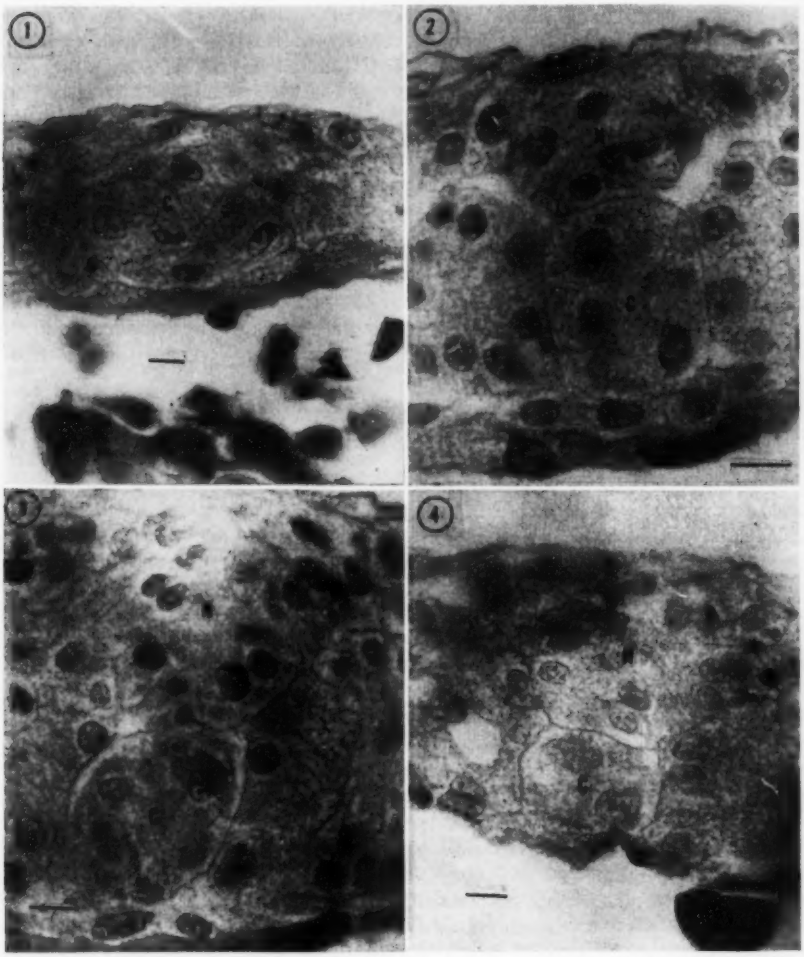


FIGURE 1. Zebra fish: cross-section at stage 15 showing the thickening in the midline of the embryo, which is the beginning of the neural primordium.

FIGURE 2. Zebra fish: cross-section at stage 16. The notochord is now distinct and the neural keel is beginning to form.

FIGURE 3. Zebra fish: cross-section at stage 17. The neural keel is enlarging.

FIGURE 4. Zebra fish cross-section at stage 17+. The keel is here quite large, but still broadly attached to the ectoderm.

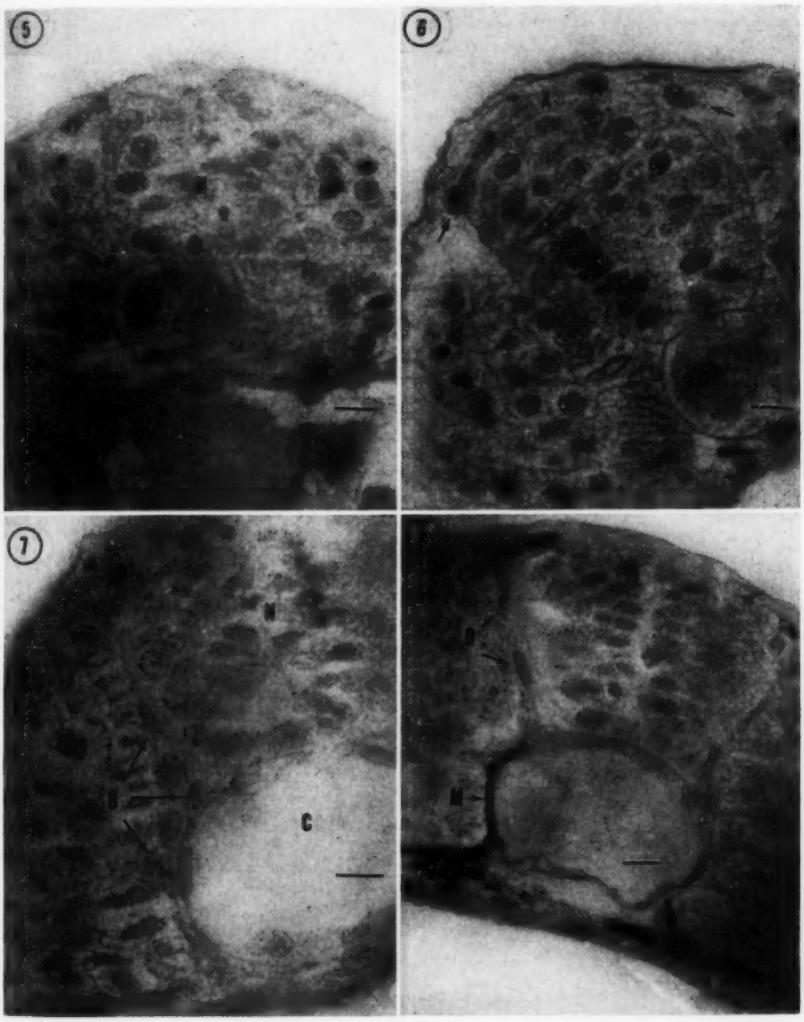


FIGURE 5. Zebra fish: cross-section at stage 18. The attachment of the keel to the ectoderm is becoming narrower.

FIGURE 6. Zebra fish: cross-section at stage 18+. This is the loose crest stage. The cells indicated (arrows) will migrate shortly.

FIGURE 7. Zebra fish: cross-section at stage 19. Some of the migrating cells (arrows) contain the first melanin granules.

FIGURE 8. Zebra fish: cross-section at stage 24. The main paths of pigment migration are outlined by melanophores.

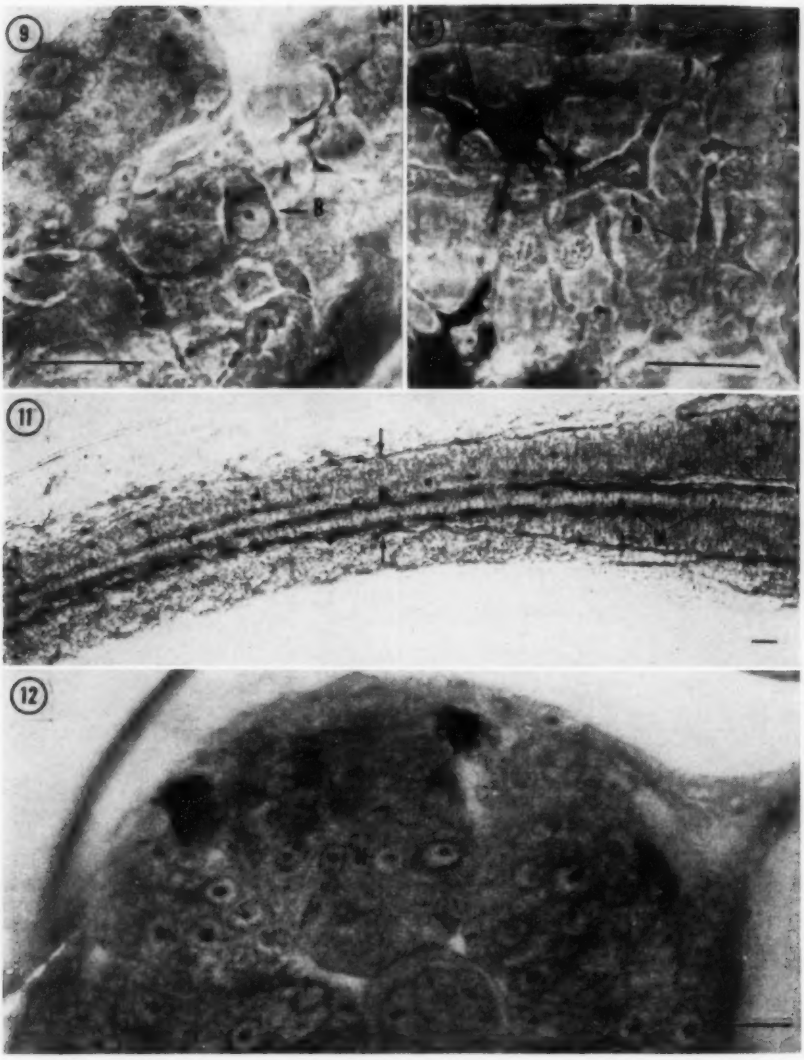


FIGURE 9. Zebra fish: phase photomicrograph at stage 24. Note the large nucleus and nucleolus of this melanoblast.

FIGURE 10. Zebra fish: phase photomicrograph at stage 24. This and the previous figure show cells in the intact, living embryo.

FIGURE 11. Dorsal aspect of a whole mount of a 30-hour cunner embryo. The width of the neural keel is indicated. The melanophores are confined to the area between the ectoderm and the keel.

FIGURE 12. Cunner: cross-section of a 20-hour embryo. Except for the presence of melanophores here, this closely resembles the loose crest stage of the zebra fish (Fig. 6).

melanogenesis there is a great increase in cytoplasmic basophilia. The nuclei triple or quadruple their volume during differentiation and contain large, densely basophilic nucleoli (Fig. 9). The cell size also increases enormously, although the very irregular shape (Fig. 10) makes calculations difficult. In general, the differentiation of the neural crest pigment cells in the zebra fish resembles that of the *Fundulus* yolk sac pigment which is not of neural crest derivation.

The pigment of the pelagic cunner egg is interesting because in similar embryos Orton (1953) and others have observed melanophores forming in the crest region well before any pigment cell migration. Figure 11 shows a 30-hour cunner embryo. The dorsal pigment has been visible for a number of hours, but the cells have not migrated. There is no pigment elsewhere in the embryo. The melanophores are still in close association with the neural tube which is beginning to form its cavity in the hind-brain region. A cross-section through an earlier cunner embryo (Fig. 12) shows the relationship of the melanophores to the neural keel. This stage resembles the loose crest stage of the zebra fish embryo.

The neural crest cells of *Fundulus* begin to migrate before melanogenesis occurs. Figures 13 to 16 show several stages in the development of the neural region of this form. By stage 14 the beginnings of the neural primordium are present. During stages 15 to 17 the keel develops rapidly (Figs. 13 and 14), and by late stage 16 or early 17 the crest becomes fairly distinct histologically. This stage may correspond with the loose crest stage of the zebra fish (Fig. 14). Between stages 17 or 18 and stage 21 crest migration begins in the trunk region (Fig. 15). Some crest migration may have started a little earlier in the head region, but it is difficult to distinguish head mesenchyme from crest cells. Sometimes at stage 18 early melanophores are visible in the head mesenchyme, possibly associated with the eye, but there are none in the trunk. Migration continues and melanization begins in the trunk during stages 19 and 20 (Fig. 16). In the discussion which follows, migrating cells are described on the yolk prior to stage 15. Some of these cells may be identified as melanoblasts at stage 16, and melanogenesis has begun in them by stage 18. Not until several hours later is the first melanin seen in the embryo proper. Thus, the yolk sac melanophores apparently are not derivatives of the neural crest.

The yolk sac melanophores

On the yolk sac of *Fundulus heteroclitus,* Stockard (1915) described wandering mesenchymal cells which eventually developed into four different cell types: black and brown pigment cells, red blood cells, and endothelial cells. These cells are readily observed with phase optics, and additional detail can be seen using the freezing-shaving technique described in this paper. The following description is based on these preparations.

The cells described by Stockard begin to migrate shortly before the closure of the blastopore (Oppenheimer stages 14 to 15) from the germ ring and possibly from the caudal mass and sides of the embryo. At these stages the cells are quite similar, and it is difficult to determine which types come from a given source. The migration continues until after the blastopore closes, at which time cells may be seen leaving the remnants of the germ ring (Fig. 17).

Stockard was not certain that his identification of these cells on the basis of

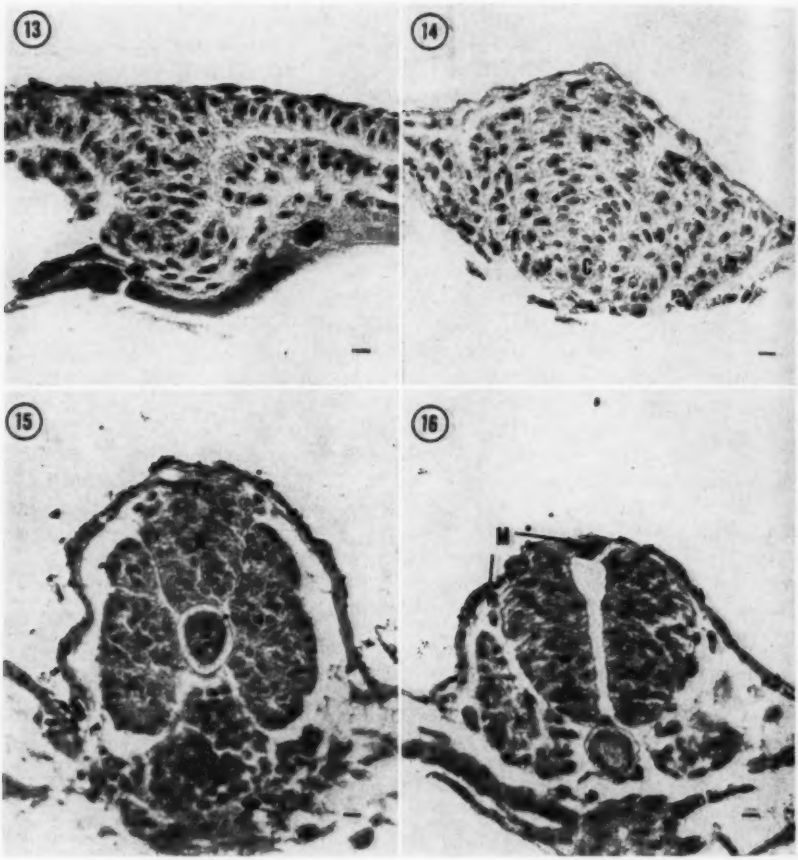


FIGURE 13. *Fundulus:* cross-section at stage 15. The neural keel is forming.

FIGURE 14. *Fundulus:* cross-section at stage 18. There is a well developed neural keel with loosening of the crest cells.

FIGURE 15. *Fundulus:* cross-section at stage 21. This is a late loose crest stage in the posterior trunk region.

FIGURE 16. *Fundulus:* cross-section at stage 21. The crest is migrating and melanophores are beginning to differentiate in this embryo. There are well developed yolk sac melanophores before this stage.

size and shape was fully warranted, and he suggested the possibility that some of the cells might be able to change from one type to another before their final differentiation. In the present study, however, it has been found that these wandering cells can be distinguished from each other on the basis of several cytological properties, and the changes in these characteristics were followed through the differentiation of the cells. (The brown pigment cells were not carefully

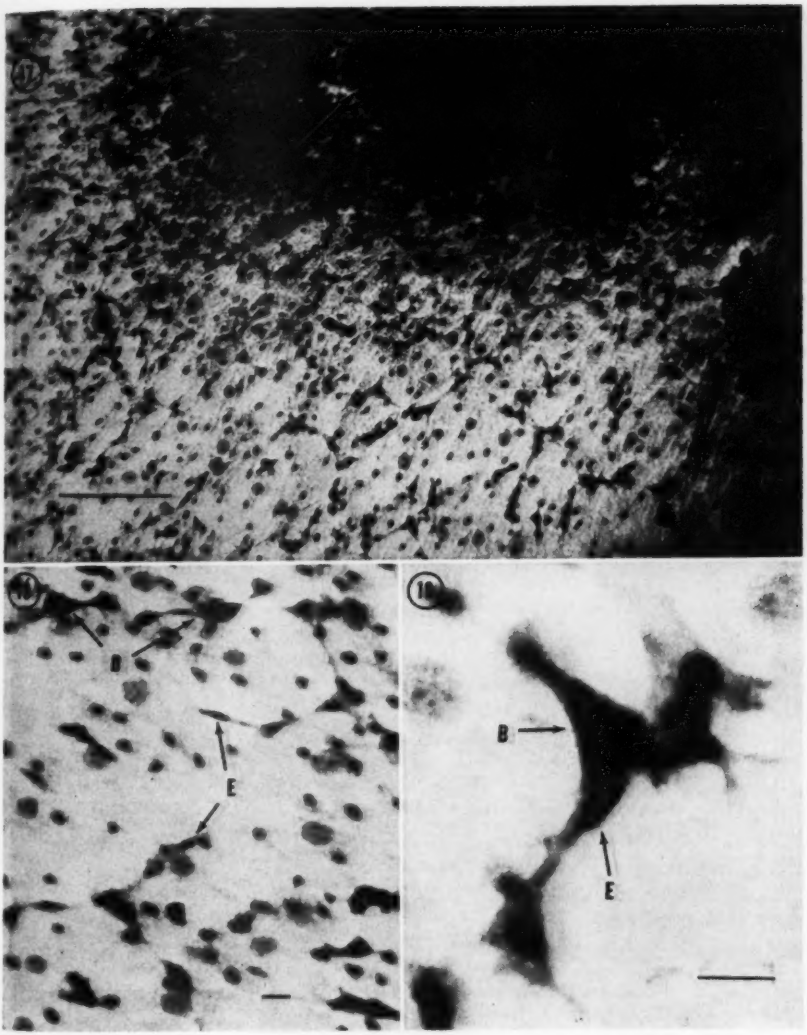


FIGURE 17. *Fundulus:* frozen-shaved preparation at stage 16. The caudal end of the embryo is at the arrow. The darkly stained cells are the wandering cells of Stockard while the lightly stained nuclei belong to the ectoderm. The scale line equals 100 μ.

FIGURE 18. *Fundulus:* frozen-shaved preparation at stage 16. The endothelial cells may be distinguished from the melanoblasts.

FIGURE 19. *Fundulus:* frozen-shaved preparation at stage 16. The large nucleus and nucleoli and the basophilic cytoplasm of the melanoblast are apparent here.

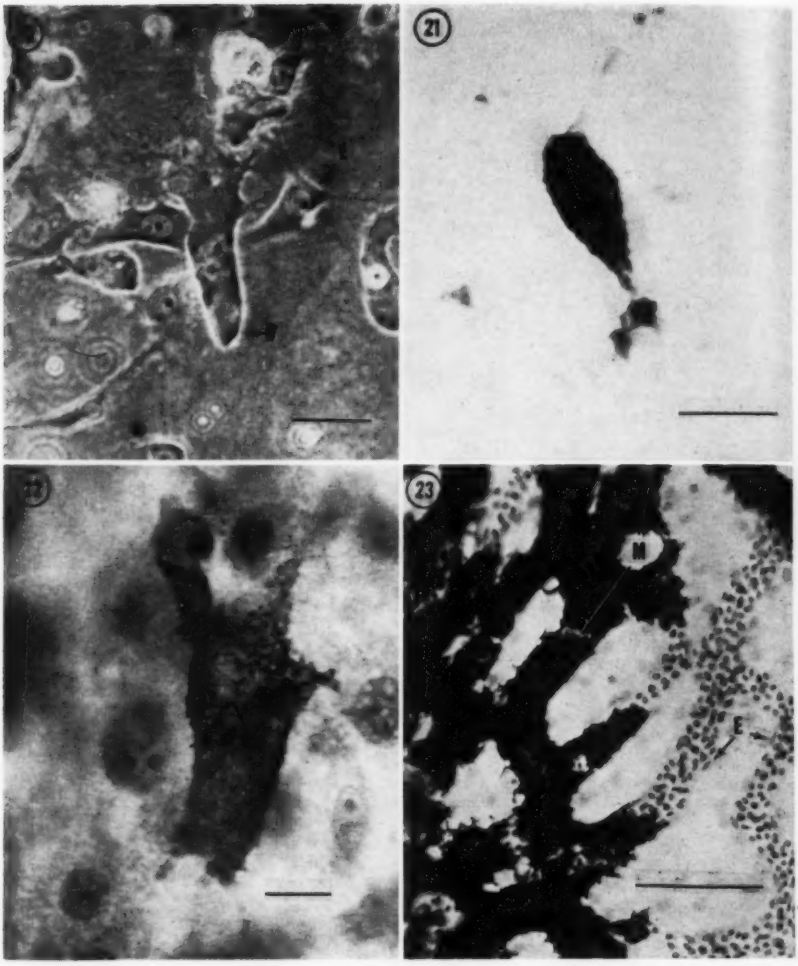


FIGURE 20. *Fundulus:* Phase photomicrograph of living stage 17 embryo. Note the cytoplasmic granularity of the melanoblast.

FIGURE 21. *Fundulus:* frozen-shaved preparation at stage 17+. Note the intense basophilia of this azure-B-stained melanoblast.

FIGURE 22. *Fundulus:* frozen-shaved preparation at stage 20. The nucleoli are still large in this early melanophore (arrows).

FIGURE 23. *Fundulus:* frozen-shaved preparation at stage 32. The extent of one melanophore is indicated. At stage 16 these cells were the same size as the blood and endothelial cells of the blood vessels which they now encompass. The scale mark is 100 μ.

followed. They seemed to arrive later and are possibly of neural crest origin.)

The migrating cells initially are spindle-shaped with lightly staining cytoplasm. Their nuclei are spherical, approximately 5 μ in diameter (70 μ^3 in volume), containing several small nucleoli. The endothelium and blood cells subsequently "lose" their nucleoli at stages 17 and 19, respectively. The blood cells become small and spherical with sharp, radiating pseudopods. They have a granular cytoplasm by stage 20, and there is little change in their nuclear volume. The endothelial cells retain their spindle shape, but stain less intensely than the original cells. Even after these cells expand to form the blood vessels of the yolk, their nuclei show little increase in volume.

The changes in the melanoblasts are quite striking, however. Figures 18 to 23 show stages in the differentiation of these cells. The most dramatic early changes are in the nuclei. The nucleoli increase in size and basophilia until the onset of melanogenesis (Figs. 19 to 22). At this time the nuclear volume is approximately 250 μ^3, and it increases further until in the fully differentiated melanophore it is almost 600 μ^3. This represents a nuclear volume increase of almost ten times over that of the initial cells. The cytoplasm of these cells becomes quite basophilic as compared with the other wandering cells (Fig. 21). A granularity also appears at stage 17 (Fig. 20), possibly representing propigment granules. The over-all cell size increases rapidly during differentiation until the final immense size of the expanded melanophore is reached (Fig. 23). These cells may thus be identified and followed with a fair degree of certainty from stage 16 through their final differentiation, and at stage 16 there is no sign of neural crest migration except rarely in the head region.

A brief search was made in the tropical fish stores and the streams of the Chicago area for other cyprinodont species which appeared to have the "non-neural crest" pigment. Simple observational criteria were used. (1) Are there numbers of relatively large wandering cells on the yolk sac before pigmentation begins? (2) Do melanophores appear evenly distributed over the yolk sac before the body pigment appears? (3) Do the yolk melanophores eventually wrap around the blood vessels of the yolk sac? The only species found which was affirmative for all three criteria was *Fundulus dispar*. *Epiplatys chaperi* was affirmative for the first two and was the only other species in which there appeared to be a secondary site of melanophore origin. Two species of *Amphyosemion* appeared to have neural crest pigment which migrated onto the yolk sac before melanogenesis began. Several other cyprinodonts had little or no yolk pigment, and no representative of the five other orders briefly studied satisfied any of the criteria. This sample, however, may be too small to make valid generalizations about the teleosts as a whole.

Discussion

In the higher classes of vertebrates where the neural tube is formed by neural folds, the neural crest might be viewed as the ectodermal tissue connecting the neural primordium with the general ectoderm. Although the neural keel of the teleosts is formed in a different manner, the crest occupies a position between it and the ectoderm. As the crest migrates, the dorsal border of the neural primordium becomes progressively more distinct until finally all attachment to the ectoderm is lost.

Since several investigators have demonstrated that the neural crest is the sole source of melanophores in most of the higher vertebrate classes (see DuShane, 1946), it is frequently stated that all vertebrate pigment cells have the same origin (see Rawles, 1948, p. 405). There is good evidence for the neural crest origin of pigment in the lampreys (Agnatha) (Newth, 1956) and fair evidence for the teleosts in general. As yet, however, there appears to be no evidence with regard to the Chondrichthyes or the Reptilia.

The relatively minor exception to the above generalization provided by *Fundulus* does, however, make a more thorough experimental study of the teleosts seem desirable. For lack of confirmation or completeness, none of the work cited constitutes adequate proof that the neural crest is the sole source of melanophores in any species of fish, while there appears to be good evidence in one species that some melanophores have a different origin.

The significance of the exception cannot be easily evaluated at present for several reasons. First, it is not known how frequently it occurs in other forms, although it does appear to be relatively rare. Secondly, the source of these exceptional melanophores has not been conclusively demonstrated, although any direct relationship with the neural crest is unlikely. Thirdly, the adaptive significance of the yolk sac pigment is unclear.

The first point above has been discussed elsewhere in this paper, and there is nothing more to add here. With regard to adaptive significance, if some fish species require either earlier pigment or pigment of different characteristics than that provided by the crest (see Rass, 1937), then the occurrence of such pigment may be of little general significance. If, instead, relatively undifferentiated tissues such as the germ ring have the potentiality of differentiating into melanophores (see Barth *et al.*, 1960), it might be found that many unrelated species have some non-neural crest pigment cells.

A certain amount of work has been done that bears on the second point above. Stockard states that most of the "wandering mesenchyme cells" come from the germ ring and its remnants after closure of the blastopore, and a smaller number come from the lateral sides of the embryo, especially at its caudal end. The differentiation of these cells does not take place until they are widely spread over the yolk sac, and it is difficult to say where they came from. Possibly they could be followed individually or traced by vital staining. Empirically, the germ ring seems the most likely source of the melanophores. It contributes most of the cells, and although there is disagreement in the literature concerning the fate of the germ ring (Lewis, 1912; Oppenheimer, 1938; Brummett, 1954), Oppenheimer's experiments demonstrate that it has broad potentialities for differentiation under experimental conditions. Perhaps it is simplest to consider the germ ring a totipotent remnant of the blastodisc which has felt comparatively little influence from the gastrulation of the embryo proper. That the embryo has little influence is borne out by the experiments of Trinkaus (1951) and by the fact that anomalous eggs may be found occasionally in which there are normal yolk sac membranes with melanophores, but no sign of embryonic structures.

It is interesting to note that the cytological aspects of differentiation are similar in both the neural crest and non-neural crest melanophores, and that in both the signs of greatest cellular activity precede the onset of melanogenesis. Quantitative

studies of cytoplasmic basophilia or nucleolar basophilia and size would be expected to follow much the same curve as the one for oxygen consumption given by Flickinger (1949)—reaching a peak at the onset of melanogenesis and falling off thereafter. Such data tend to indicate that in the melanophore, at least, the processes of differentiation may take more cellular "effort" than the elaboration of the materials characteristic of the differentiated cell.

Much of the material in this paper was submitted as a dissertation for the degree of Master of Science at the University of Chicago. The author wishes to express his thanks to his sponsor, Dr. Hewson Swift, for help on many aspects of this study.

Summary

In this study, three species of fish which differ in the developmental histories of their pigment cells have been used—the zebra fish (*Brachydanio rerio*), the cunner (*Tautogolabrus adspersus*), and *Fundulus heteroclitus*. Their melanophores have been studied with respect to their origins and subsequent histories, including some of the cytological events occurring during differentiation.

1. In the teleosts, the neural crest has been identified as the tissue connecting the neural keel with the overlying ectoderm. During development, this tissue loosens, and the cells migrate from this region. In some of these cells melanogenesis has already begun.

2. Differentiated melanophores are demonstrated in the neural crest of the cunner embryo well before migration occurs.

3. It is concluded that the normal embryonic pigment of the teleosts has its origin in the neural crest, as in other vertebrates.

4. In some fish, notably in *Fundulus*, the pigment cells of the yolk sac are not of neural crest origin. Instead they appear to arise from the extra-embryonic germ ring. These cells may be identified on the yolk sac several hours before there is histological evidence of crest migration.

5. Many of the cytological aspects of differentiation are similar in the melanophores, regardless of their site of origin.

LITERATURE CITED

Axelrod, H. R., and L. P. Schultz, 1955. Handbook of Tropical Aquarium Fishes. McGraw-Hill Co., New York.

Bancroft, F. W., 1912. Heredity of pigmentation in *Fundulus* hybrids. *J. Exp. Zool.*, **12**: 153–178.

Barth, L. J., L. G. Barth and I. Nelson, 1960. Induction of pigment cells from the presumptive epidermis of *Rana pipiens* gastrula. *Anat. Rec.*, **137**: 337–338.

Borcéa, M. I., 1909. Sur l'origine du coeur, des cellules vasculaires migratrices et des cellules pigmentaires chez les téléostans. *C. R. Acad. Sci., Paris*, **149**: 688–689.

Brummett, A. R., 1954. The relationship of the germ ring to the formation of the tail bud in *Fundulus*. *J. Exp. Zool.*, **125**: 447–481.

Costello, D. P., M. E. Davidson, A. Eggers, M. H. Fox and C. Henley, 1957. Methods for Obtaining and Handling Marine Eggs and Embryos. Marine Biological Laboratory, Woods Hole, Mass.

DuShane, G. P., 1935. An experimental study of the origin of pigment cells in Amphibia. *J. Exp. Zool.*, **72**: 1–31.

DuShane, G. P., 1946. The development of pigment cells in vertebrates. *In:* Biology of the Melanomas. *Spec. Publ. N. Y. Acad. Sci.*, **4**: 1–15.

Flickinger, R. E., 1949. A study of the metabolism of the amphibian neural crest cells during their migration and pigmentation *in vitro*. *J. Exp. Zool.*, **112**: 465–484.

Gilson, A. S., 1926. Melanophores in developing and adult *Fundulus*. *J. Exp. Zool.*, **45**: 415–457.

Goodrich, H. B., 1950. Problems of origin and migration of pigment cells in fish. *Zoologica*, **35**: 17–19.

Hisaoka, H. K., and H. I. Battle, 1958. The normal stages of the zebra fish. *J. Morphol.*, **102**: 311–321.

Humm, D. G., and R. S. Young, 1956. The embryological origin of pigment cells in platyfish-swordtail hybrids. *Zoologica*, **41**: 1–10.

Kajishima, T., 1958. Regional differences in pigment cell formation of the embryonic shield of the goldfish. *Embryologia*, **4**: 133–147.

Lewis, W. H., 1912. Experiments on localization and regeneration in the embryonic shield and germ ring of a teleost fish, *Fundulus*. *Anat. Rec.*, **6**: 325–334.

Lopashov, G. V., 1944. Origin of pigment cells and visceral cartilages in teleosts. *C. R. (Doklady) Acad. Sci. U. R. S. S.*, **44**: 169–172.

Newth, D. R., 1951. Experiments on the neural crest of the lamprey embryo. *J. Exp. Biol.*, **28**: 247–260.

Newth, D. R., 1956. On the neural crest of the lamprey embryo. *J. Embryol. Exp. Morphol.*, **4**: 358–375.

Oppenheimer, J. M., 1937. The normal stages of *Fundulus heteroclitus*. *Anat. Rec.*, **68**: 1–15.

Oppenheimer, J. M., 1938. Potencies for differentiation in the teleostean germ ring. *J. Exp. Zool.*, **79**: 185–212.

Oppenheimer, J. M., 1949. Atypical pigment cell differentiation in embryonic teleostean grafts and isolates. *Proc. Nat. Acad. Sci.*, **35**: 709–712.

Orton, G. L., 1953. Development and migration of pigment cells in some teleost fishes. *J. Morphol.*, **93**: 69–89.

Rass, T. S., 1937. Pigmentation of embryos and larvae in the herrings as an adaptation to a pelagic mode of life. *Bull. Soc. Nat. Moscow, N. S.*, **46**: 163–164.

Rawles, M. E., 1948. Origin of melanophores and their role in the development of color patterns in vertebrates. *Physiol. Rev.*, **28**: 383–408.

Russel, A., 1939. Pigment inheritance in *Fundulus-Scomber* hybrids. *Biol. Bull.*, **77**: 423–431.

Stockard, C. R., 1915. A study of wandering mesenchymal cells on the living yolk sac and their developmental products: chromatophores, vascular endothelium, and blood cells. *Amer. J. Anat.*, **18**: 525–594.

Trinkaus, J. P., 1951. Analysis of blastoderm expansion of the egg of *Fundulus heteroclitus*. *Anat. Rec.*, **111**: 550–551.

CERCARIA DIPTEROCERCA MILLER AND NORTHUP, 1926 AND STEPHANOSTOMUM DENTATUM (LINTON, 1900) MANTER, 1931 [1]

HORACE W. STUNKARD

The American Museum of Natural History, New York 24, N. Y., and the Marine Biological Laboratory, Woods Hole, Massachusetts [2]

After a preliminary survey had shown that *Nassa obsoleta* is the most heavily parasitized gastropod in the Woods Hole region, Miller and Northup (1926) reported on a study conducted for one year to determine seasonal infection of this snail by larval trematodes. They examined 8875 individuals, first by isolation for 48 hours to determine those from which mature cercariae were emerging, and subsequently by crushing the snails individually and examining the tissues under a binocular microscope for infection by the asexual generations. They recorded total and seasonal infection by five species, all described as new to science, and named *Cercaria setiferoides, Cercaria quissetensis, Cercaria variglandis, Cercaria dipterocerca,* and *Cercariaeum lintoni.* The last species had been described but not named by Linton (1915).

Later studies have traced the life-cycles and systematic relations of these cercariae. Martin (1938) showed that *C. setiferoides* encysts in turbellarians and spionid annelids and becomes mature in the sand-dab, *Hippoglossoides platessoides,* and small flounders (not specifically identified but probably *Pseudopleuronectes americanus* or *Paralichthys dentatus*). The adults were described as *Lepocreadium setiferoides* (Miller and Northup). Stunkard (1938a) worked out the life-cycle of *C. quissetensis;* the cercariae encyst in marine mollusks and become sexually mature in the herring gull, *Larus argentatus.* They were described as *Himasthla quissetensis* (Miller and Northup, 1926). Stunkard (1938b) found that *C. lintoni* had been described and named *Distomum lasium* by Leidy (1891); that the metacercariae encyst in polychaete annelids and mature in fishes. The worms were assigned to *Zoogonus rubellus* (Olsson, 1898) Odhner, 1902, but after study of the European species, whose metacercariae were found in sea-urchins, Stunkard (1941) recognized bionomic and morphological differences between the European and American species and listed the adults of *C. lintoni* as *Zoogonus lasius* (Leidy, 1891). Stunkard and Hinchliffe (1952) discovered that *C. variglandis* is the causative agent of "swimmers' itch" of oceanic beaches in New England and the adults proved to be identical with avian blood-flukes that had been described by Price (1929) as *Microbilharzia chapini.* Penner (1953) suppressed *Microbilharzia* as a synonym of *Austrobilharzia* Johnston, 1917 and listed the species as *Austrobilharzia variglandis* (Miller and Northup, 1926). The fifth species, *Cercaria*

[1] This investigation was supported in part by contract between the Marine Biological Laboratory and the ONR; Nonr-1497 (00).

[2] Mailing address: The American Museum of Natural History, Central Park West at 79th Street, New York 24, N. Y.

dipterocerca, has been under observation for several summers and although experimental infection of the final host has not yet been accomplished, development of the metacercarial stage in experimental infections permits its identification as a species of the genus *Stephanostomum*. In number and shape of peristomial spines and in general morphology, the metacercariae manifest striking agreement with juvenile and mature specimens of *Stephanostomum dentatum* (Linton, 1900) Manter, 1931. Although experimental proof has not been obtained, the agreement suggests that *C. dipterocerca* may be the larval stage of *Stephanostomum dentatum*.

The genus *Stephanostomum* was erected by Looss (1899) with *S. cesticillum* (Molin, 1858) as type species. The name *Stephanostoma* had previously been applied to a dipteran larva by Lenz (1794) and to a sipunculid worm by Danielssen and Koren (1880). Believing the name to be preoccupied, Looss (1900) renamed the genus *Stephanochasmus*. Manter (1934, 1940) noted that according to a Recommendation under Article 36 of the International Rules of Zoological Nomenclature, slight differences in spelling do not invalidate a generic name, and accordingly he restored *Stephanostomum* with *Stephanochasmus* as a synonym. Linton (1910) described *Stephanochasmus casus* n. sp., and proposed a new genus and species, *Lechradena edentula*, for a single worm from the gray snapper, *Neomaenis* (= *Lutianus*) *griseus*. Pratt (1916) gave a detailed description of *S. casus* and noted, p. 229, "all the spines are very easily lost by maceration." Referring to the worm described as *L. edentula*, Pratt stated that except for the absence of spines, its structure agrees in every detail with that of *S. casus* and that the spines had probably been lost. A somewhat similar situation appertains in the case of *Distomum osculatum* Looss, 1901, a species described from two specimens and named by Poche (1926) as type of a new genus, *Tormopsolus*. The specimens lack peristomial spines but otherwise are so similar to *Stephanostomum caducum* that Manter (1934) suspected that the spines had been lost and that the worms belong to a species of *Stephanostomum*. The same opinion was expressed by Wolfgang (1955b). McFarlane (1934) reported that in *Stephanostomum casum* (Linton, 1910) McFarlane, 1934, the digestive ceca communicate with the excretory vesicle to form a uroproct. To contain this species and another, similarly provided with a uroproct and which he described as new, Yamaguti (1934) erected the genus *Echinostephanus* but Ward (1938) suppressed *Echinostephanus* as a synonym of *Stephanochasmus*. A single specimen taken from *Sphyraena barracuda* was described by Pérez Vigueras (1942) as *Monorchistephanostomum gracile* n.g., n. sp., but Yamaguti (1958) suppressed *Monorchistephanostomum* as another synonym of *Stephanostomum*. These instances show how the literature is encumbered by the unnecessary and unwise erection of new taxonomic units for single specimens or otherwise inadequate material.

The genus has a wide distribution; its members infect marine teleosts, particularly those of warmer water. Species have been reported from the Atlantic coast of Europe and North America, the Black Sea and the Mediterranean, Bermuda, Tortugas, the Galapagos Islands, the west coast of both South and North America, New Zealand, the Indian Ocean, and the coasts of China and Japan. Specific determination is based on the number, shape and position of peristomial spines, relative size of suckers, extent and arrangement of the vitellaria, length of the cirrus-sac and genital atrium, distances between the gonads, presence or absence of

a uroproct, and size of eggs. But the extent of variation in single species is virtually unknown and the number of valid species is still uncertain. Manter (1947) listed nine species from fishes taken at Tortugas, Florida. ***Stephanostomum longisomum*** Manter, 1940 and *Stephanostomum filiforme* Linton, 1940 were suppressed as synonyms of *Stephanostomum ditrematis* (Yamaguti, 1939) and he noted that *Stephanostomum pagrosomi* (Yamaguti, 1939) differs from *S. dentatum* (Linton, 1900) only in slightly larger acetabulum and slightly larger eggs. Manter and Van Cleave (1951) recognized twenty-eight species and devised a key for their identification. Three worms from the California halibut, *Paralichthys californicus*, were identified as *S. dentatum*, and *S. pagrosomi* was definitely relegated to synonymy. Montgomery (1957) reported *S. dentatum* from *P. californicus* at La Jolla, California, and stated that the parasite has been taken also at Woods Hole, Massachusetts; Beaufort, North Carolina; Tortugas, Florida; and the Inland Sea, Japan. Caballero (1952) gave a monographic account of the genus, associated certain larval forms with their corresponding adults, corrected items of synonymy, and arranged a key for thirty species. Yamaguti (1958) listed forty-two species in the genus.

Encysted larvae, referable to *Stephanostomum*, were reported by Krøyer (1838–1840) from *Platessa vulgaris* and *Rhombus maximus*. Specimens from the same hosts and presumably identical, were described by Dujardin (1845) as *Distoma hystrix*. Filippi (1855) described *Cercaria echinocerca* from *Buccinum linnaei* as the probable larval stage of *D. hystrix*, and *Moulinié* (1856) discussed the possibility in his review of reproduction in endoparasitic trematodes. Olsson (1868) reported *D. hystrix* from *Pleuronectes limanda*. Stossich (1883) described encysted metacercariae found in *Gobius jozo* at Triest under the name *Distomum valdeinflatum*. Three years later he transferred the species to *Echinostoma*, and Looss (1899) identified both *D. hystrix* and *D. valdeinflatum* as species of *Stephanostomum*. Linton (1898) referred encysted distomes, usually attached by a slender peduncle to the peritoneal wall of the file-fish, *Alutera schoepfii* (= *Ceratacanthus schoepfii*), to *D. valdeinflatum*. He noted that these metacercariae resembled adult *Distomum tenue* Linton, 1898, found in *Roccus lineatus*, and suggested that the two may be identical. Later, Linton (1940) identified as ***Stephanostomum valdeinflatum*** certain immature, encysted distomes taken at various times from the file-fish (*Ceratacanthus schoepfii*), silversides (*Menidia notata*), toadfish (*Opsanus tau*) and a single macerated, mature specimen (Helminth. Coll., U. S. Natl. Museum, No. 8210) from the intestine of the sunfish (*Mola mola*). Caballero (1952) suggested that certain of the encysted larvae should be referred to *Stephanostomum imparispine* (Linton, 1905) Manter, 1940, a species initially described from *Rachycentron canadus* taken at Beaufort, North Carolina, and reported from the same host by Sogandares-Bernal and Hutton (1959b). But the mature specimen from *M. mola* can not be assigned to *S. imparispine;* it differs greatly in size, in relative size and position of the testes, and in extent of the vitellaria. The reported seminal receptacle is actually a bulbous, spermatozoa-filled expansion of the initial portion of the uterus rather than a preootypic receptacle. The worm more closely resembles *S. dentatum*. Encysted larvae, identified as *S. hystrix*, were found by Stafford (1904) in cysts on fins of the winter flounder, *Pseudopleuronectes americanus*, and free in the

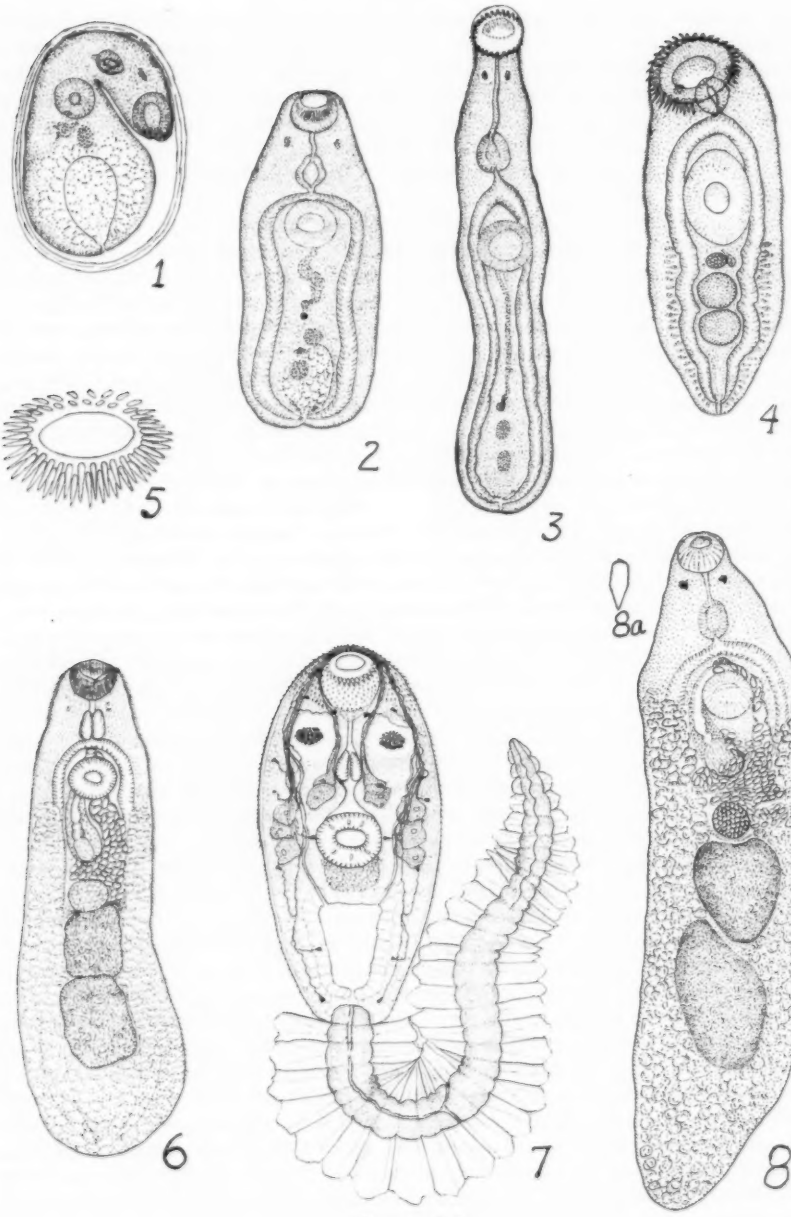
stomach of *Lophius piscatorius* which also contained two partially digested specimens of the dab, *Limanda ferruginea*.

Other encysted larvae from various parts of the world have been referred to *Stephanostomum* on the ground of morphological similarity but without experimental confirmation. They were found in Britain by Johnstone (1905), Lebour (1908), Nicoll and Small (1909) and Nicoll (1910); in Ceylon by Lühe (1906), in Japan by Yamaguti (1934, 1937) and in Florida by Sogandares-Bernal and Hutton (1959a). Martin (1939) reported the first experimentally demonstrated life-cycle in the genus. He showed that sticky-tailed, stylet-bearing, ocellate cercariae from rediae in *Nassa obsoleta* encyst and develop to metacercariae in the mesenteries and liver of *Menidia* (*Menidia*) *notata* and continue development to nearly mature adult worms in the intestine of young puffers, *Spheroides maculatus*. The worms were identified as *Stephanostomum tenue* (Linton, 1898) Martin, 1938, a species described originally from the striped bass, *Roccus lineatus*, at Woods Hole, Massachusetts, and subsequently (Linton, 1940) from the sand-launce (*Ammodytes americanus*), the sea-raven (*Hemitripterus americanus*), the kingfish (*Menticirrhus saxatilis*), the white perch (*Morone americana*), the striped bass (*Roccus saxatilis*), and the toadfish (*Opsanus tau*). Margarita Bravo (1956) reported *S. tenue* from the intestine of *Trachurops crumenophthalma* taken on the Pacific coast of Mexico.

The life-cycle of a second species, *Stephanostomum baccatum*, was reported by Wolfgang (1954, 1955a). According to the report, ophthalmoxiphidiocercous cercariae, "secondarily" lacking stylets, develop in rediae in *Buccinum undatum* and *Neptunea decemcostatum*, encyst as metacercariae in the skin and somatic muscles of pleuronectid fishes and occur as adults in the rectum of *Hemitripterus americanus* and other fishes. A description was given of all stages in the cycle but there are disturbing inconsistencies in the account which may invalidate the conclusions. *Stephanochasmus baccatus* (= *Stephanostomum baccatum*) was described by Nicoll (1907) on a single specimen from the intestine of *Hippoglossus hippoglossus*. The description was adequate to validate the species and was supported by a good figure. The species was reported by Manter (1926) from the same host taken at Mount Desert Island, Maine. Stafford (1904) recorded *Stephanostomum sobrinum* (Levinsen, 1881) Looss, 1901 from the sea-raven (*Hemitripterus americanus*), the arctic eelpout (*Lycodes* sp.), and the wrymouth (*Cryptoacanthodes maculatus*). Caballero (1952) intimated that the specimens are probably not identical with *S. sobrinum* and suggested that they may belong to *S. tenue*. After examination of Stafford's specimens, Wolfgang (1955a) stated that they are *S. baccatum*. Wolfgang studied approximately 1000 specimens, taken from a variety of hosts, but data from literature and from observation are intermingled and frequently it is difficult if not impossible to distinguish between them. He reported the worms from the lower intestine and rectum and listed as final hosts the three species named by Stafford and also *H. hippoglossus* and the short-horned sculpin (*Myoxocephalus scorpius*). The distribution of the parasite was given as across the North Atlantic Ocean from Maine to Nova Scotia, Newfoundland, Greenland and the North Sea coast of Europe. But Wolfgang was doubtful of the validity of *S. baccatum* since he stated (1955a, p. 119), "Levinsen inadequately described *S. sobrinum* from *Cottus scorpius* in Greenland waters, and Odhner (1905) reported but did not redescribe it from the same host in unspecified

arctic waters. Although Levinsen's description of *S. sobrinum* is vague it agrees essentially with that of *S. baccatum*. In view of the hosts and the distribution of the parasite, *S. sobrinum* and *S. baccatum* are probably conspecific." Stafford may have been correct in his determination and if the two species are identical, *S. baccatum* disappears as a synonym of *S. sobrinum*. Furthermore, Wolfgang stated (1955b, p. 135), "that *S. baccatum* shows no valid differences from *S. triglae*." If these species are conspecific, *S. triglae* Lebour, 1908 also would be suppressed as a synonym.

Although Wolfgang gave descriptions of the successive stages in the life-cycle of *S. baccatum*, there is no assurance that the cycle was actually completed in experimental hosts. The methods were so lacking in control that opportunity for confusion was obvious. There is virtually no evidence that the cercariae taken by dissection from *Buccinum undatum* and *Neptunea decemcostatum* develop into the metacercariae found encysted in the flounders. Moreover, proof of the identity of the metacercariae encysted in winter flounders, *Pseudopleuronectes americanus*, and the adults of *S. baccatum* was based on infections resulting from the following procedure, (1954, p. 965), "Gelatin capsules were filled with cysts taken from fins and muscles of the winter flounder and placed in the siphons of clams which were then fed to the fish." The author admitted (p. 965), "Although several hundred cysts were used for each dose, no comparison between numbers of cysts introduced into experimental hosts and numbers of adult worms recovered could be made because their viability may have been impaired by dissection." But the cysts of *Stephanostomum* are so tough and resistant that it is difficult to see how the infectivity of the larvae could be impaired during dissection. Furthermore, it is apparent from the report that the fishes used for experiment were already infected and as many as 567 worms were taken from a sea-raven collected near Clam Cove, Deer Island, N. B. The data presented in Table I (1954, p. 966) show that after feeding several hundred cysts in each dose, the heaviest infection (123 adult worms) was found in a sea-raven dissected four days after feeding, at which time the metacercariae of experimental infection would not yet be adult, and that the number of adult worms decreased with the length of time after feeding to a single worm in an eelpout after twenty-two days, the longest time recorded. These data strongly suggest that natural infections harbored by the experimental fishes were gradually lost during captivity and there is no proof that the worms recovered at autopsy were derived from the cysts that had been fed in the experiment. But the most disturbing inconsistencies are found in the morphological section of the paper. As described, the cercariae show no significant differences from *C. neptuneae* Lebour, 1912 from the same and related hosts, and the two may be identical. An excretory duct is described as extending posteriad from the bladder and terminating in a pore at the tip of the tail. Such a condition is so unusual in a cercaria of this type that the observation is open to question. Moreover, in the cercaria the excretory collecting ducts are portrayed as mesostomate, *i.e.*, they bifurcate behind the acetabulum to give rise to anterior and posterior branches, whereas in the metacercaria they are represented as stenostomate, extending forward without branching to the level of the eye-spots. The latter condition is characteristic of species in the genus *Stephanostomum*. If the descriptions are correct, it is obvious that the cercaria and metacercaria belong to different taxonomic groups and can not be stages in the life-cycle of a single species.



FIGURES 1–8a.

Material and Methods

Nassa obsoleta, collected in the Woods Hole area, were isolated to find those which were shedding *Cercaria dipterocerca*. The incidence of infection was about 0.2% of the snails taken at the Sheep-Pen Cove on Nonamesset Island; less at other places. Miller and Northup (1926) reported a total of only four infections in the examination of 8875 snails collected at Quisset harbor. When snails are kept for long periods in fingerbowls, the number of cercariae released each day gradually diminishes, and after 12 to 16 weeks shedding may be stopped completely. But although reproduction of the parasite slows down or ceases temporarily, the infection remains in dormant condition and becomes active when the snail again has an abundance of food. It appears that a parasitized snail carries the infection indefinitely and that the parasite undergoes periods of interrupted activity during the winter or whenever the mollusk lacks sufficient nourishment to maintain both its basic metabolism and reproduction of the parasite.

Since the cercariae are provided with penetration glands, they were added to small aquaria with possible invertebrate and vertebrate hosts. They invaded small *Fundulus heteroclitus* and *Menidia menidia* but the fishes often harbored natural infections which could be confused with experimental ones. Dr. Evelyn Shaw reared *M. menidia* from eggs at the Marine Biological Laboratory, and kindly provided small fishes that had not been exposed to infection and therefore afforded absolute control of the experiments. Grateful acknowledgment is made here to Dr. Shaw. The small fishes were fed brine shrimp, *Artemia salina*. The cercariae penetrated the tissues and encysted in the throat, in the mesenteries and in the liver. They were found also under the skin and in the muscles, but the cysts were chiefly in the viscera and apparently the cercariae were taken into the mouths of the fishes with water for respiration. Dissection of these *M. menidia*

Figure 1. Metacercaria, in cyst 0.28 mm. long, from laboratory-reared *Menidia menidia*, exposed to *Cercaria dipterocerca*.

Figure 2. Metacercaria, from laboratory-reared *M. menidia* exposed to *C. dipterocerca*, released from cyst and pressed to study excretory system; worm as fixed, stained and mounted is 0.62 mm. long. This worm has 54 peristomial spines.

Figure 3. Metacercaria, identified as *Stephanostomum tenue*, released from cyst from naturally infected *Fundulus heteroclitus*, Woods Hole, Mass. Specimen fixed, stained and mounted is 1.69 mm. long. This worm has 42 peristomial spines; compared with Figure 2, note length of prepharynx and location of gonads.

Figure 4. Smallest of 12 worms on slide No. 8199, Helminthological Collection, U. S. Nat. Museum. Material of *Stephanostomum dentatum* collected 6 October 1911 by Vinal Edwards from *Paralichthys dentatus* at Woods Hole, Mass., ventral view. The worm is somewhat contracted, the anterior end curved ventrally and as mounted is 0.70 mm. long. This worm has 54 peristomial spines.

Figure 5. The peristomial spines of the largest specimen, 2.31 mm. long, on slide No. 8199, Helminth. Coll., U. S. Nat. Museum. This worm has 54 peristomial spines.

Figure 6. The largest specimen collected by the writer, *S. dentatum* from *P. dentatus*, at Woods Hole. Fixed under coverglass pressure, stained and mounted, it is 2.79 mm. long.

Figure 7. *Cercaria dipterocerca*, from *Nassa obsoleta*, ventral view, the portion of the excretory system in the tail undergoing regression.

Figure 8. Specimen, 3.5 mm. long, on slide No. 8200, Helminth. Coll., U. S. Nat. Museum. Collected 5 September 1919 from *P. dentatus;* identified as *S. dentatum* and figured in Linton (1940), Plate 3, Figure 25. Only a few peristomial spines remain; they are broad, flat, scale-like.

Figure 8a. Peristomial spine from worm shown in Figure 8.

at intervals during the ensuing six weeks yielded a series of developing metacercariae, from recently encysted larvae to mature specimens indistinguishable from those found in natural infections of *M. menidia* and *F. heteroclitus* taken in the Woods Hole area. Most of the natural infections, however, were *Stephanostomum tenue* (Fig. 3) which can be distinguished from *S. dentatum* because the circumoral spines are fewer in number and different in shape. Other differences are apparent from comparison of Figures 2 and 3.

Juvenile and adult specimens of *S. dentatum* have been found as natural infections of *Paralichthys dentatus* taken at Woods Hole. Small and medium sized representatives of this species have been kept for several weeks in the laboratory. But attempts to infect final hosts, *P. dentatus* and other flatfish, have so far not been successful, since the fishes have consistently refused to eat in the aquaria. When a larger aquarium with colder water is available, it should be possible to complete the study and obtain experimental infection of final hosts that had been held in a control tank for several weeks to insure that all worms of natural infection would be sexually mature and that those of experimental infection could be positively identified. Experimental infection of the snail host must await a supply of eggs and miracidia from infected fishes.

Study of the specimens of *Stephanostomum* on deposit in the Helminthological Collection of the U. S. National Museum was afforded through the kindness of Dr. Allen McIntosh, to whom grateful acknowledgment is made. Many of the worms were not suitable for specific identification, but slide No. 8199 contained twelve specimens of *S. dentatum*, collected October 6, 1911, by Vinal Edwards from *Paralichthys dentatus* at Woods Hole, Massachusetts. The specimens ranged from juvenile worms (Fig. 4) to fully mature, gravid individuals, and provided opportunity for comparison of these worms with metacercariae recovered from experimental hosts after exposure to *Cercaria dipterocerca*.

Descriptions

Cercaria dipterocerca

Redia

The rediae were described briefly and figured by Miller and Northup (1926). The hemal sinuses of the snail frequently contain scores of small rediae 0.10 to 0.20 mm. in length, similar to the one shown in Figure 4 of Miller and Northup. In these small rediae the body is colorless, the pharynx 0.02 to 0.03 mm. in diameter, the digestive cecum is saccate, about one-half the length of the body, and frequently it contains orange-colored droplets. These larvae are very mobile, moving easily by alternate contractions of annular and longitudinal muscles, anchoring one region of the body while advancing another. There are no feet, specialized portions of the body-wall, which may be protruded to serve as holdfasts. Discovery of a small redia with a contained daughter-redia demonstrates more than one redial generation and the large number of small rediae suggests repeated generations of daughter-rediae.

Larger rediae, containing germ-balls and one or more cercariae, measure 0.25 to 0.75 mm. in length and 0.07 to 0.14 mm. in width. Old ones, empty except for a few germ-balls, are seen occasionally; they may measure up to 1.25

mm. in length, with long, pointed, posterior ends. The average measurement given by Miller and Northup is larger than found in the present study and may have been made on specimens under considerable coverglass pressure. In general, the rediae are oval to cylindrical, often attenuated at one or both ends. Frequently the anterior end is prolonged by a long, slender, neck-like region, about one-half the diameter of the succeeding portion of the body. Contraction of circular muscles may produce an annulate appearance or the formation of two or more wider regions separated by narrower ones. The pharynx is oval to spherical and 0.025 to 0.048 mm. in diameter; its wall is about 0.011 mm. thick. With increasing size and the maturity of increasing numbers of cercariae, the digestive ceca become relatively shorter, the body-walls become stretched and thinner, and the rediae less active. The wall contains yellow to orange droplets, one to four microns in diameter, usually associated in larger oval or circular masses, ten to fifteen microns in diameter. These masses easily dissociate. The cecum contains material, orange to greenish to blackish in color. A birth pore is present, located about the length of the pharynx behind that sucker. The cercariae move about freely in the cavity of the redia.

Cercaria (Figure 7)

Examination of unstained living specimens and others, after staining with vital dyes and after fixation, staining and mounting as permanent preparations, confirms the original description in large part and adds details of the excretory system. The two pairs of glands which stain with neutral red, situated lateral and posterior to the pharynx, were observed. There are apparently three, rather than four, penetration glands situated at either side of the acetabulum. The cells are lobed and partially overlap one another, which renders delineation difficult. Only three ducts were identified although ducts, when empty, are not visible. The cells of the third group, labelled *C* in the figure of Miller and Northup, were not observed.

Bodies of normally emerged cercariae, fixed without pressure in swirling, hot Duboscq-Brasil fluid and stained with Semichon technique, measure 0.135 to 0.145 mm. in length and 0.046 to 0.053 mm. in width. The tails are 0.220 to 0.240 mm. in length and 0.016 to 0.020 mm. wide at the base. The acetabulum is 0.023 to 0.024 mm. in diameter; the oral sucker is slightly larger, 0.024 to 0.025 mm. in diameter; the pharynx is oval to pyriform, usually wider posteriorly, 0.01 to 0.014 mm. in diameter; and the ceca are not developed. Above and behind the acetabulum there is a mass of deeply staining germinal cells, rudiments of the reproductive organs and ducts.

Young cercariae in rediae lack spines, there are no membranous fins on the tail, the ocelli are small and the two excretory pores on the sides of the tail are clearly visible. Indeed, in very young specimens the excretory ducts are not fused in the base of the tail. In such specimens the acetabulum is situated more posteriorly, about one-fourth of the body length from the posterior end; it gradually is moved anteriad as the postacetabular portion of the larva grows faster than the anterior portion.

Normally emerged cercariae are photonegative; they swim rapidly, tail in advance, with the body bent ventrally. The body may extend to a length of 0.240

mm. with a corresponding reduction in width. The tail is subterminal in attachment; it may be extended to a length of 0.340 mm. and the tip may be protruded as a small papilla. The central portion of the tail is 0.018 to 0.025 mm. wide near the base and the lateral cuticular fins are about the same width. The lateral fins have 30 to 36 undulatory plications, the crests of which may simulate setae. The median fin is narrower than the lateral ones; it extends along the ventral side of the distal one-fourth of the tail and forward for about one-third of that distance on the dorsal side. In a fixed and stained specimen, 44 nuclei were counted in the tail. The cuticula of the body, but not of the tail, is covered with spines. There is a narrow spineless area around the mouth and then two rows of alternating peristomial spines, typically 27 in each row. Behind these collar-spines the spines are slightly recurved with broad bases, gradually becoming narrower and smaller posteriorly. There is a pair of conspicuous ocelli or eye-spots, 0.009 to 0.012 mm. in diameter, situated above the lateral ends of the commissure of the nervous system. A very large number of pigment granules surrounds each lenticular cup. When the body is extended, the ocelli are circular but when it is retracted the ocelli are oval, wider than long.

The excretory vesicle is V-shaped when filled and Y-shaped with short arms when empty. It has a definite epithelial wall and is surrounded by cystogenous cells. It is formed by the coalescence and fusion of the excretory ducts of each side, although in young cercariae the two ducts are distinct and open on the sides of the tail, some one-fourth to one-fifth of its length from the base. The ducts in the tail subsequently fuse and regress, so that in emerged cercariae there may be only lateral excretory pores at the junction of the body and tail. The collecting ducts pass forward, ventral to the ducts of the penetration glands, from the anterolateral faces of the excretory vesicle to the level of the ocelli where they turn backward and each gives off a branch which runs in front of the ocellus and divides into three capillaries that drain the three flame-cells situated in the region of the oral sucker. The recurrent tubule passes backward and gives off a second branch that divides to supply the three flame-cells in the preacetabular and acetabular area and then continues posteriad to break up into three capillaries that drain three flame-cells in the postacetabular area. There are tufts of long cilia in the collecting ducts; movement of particles can often be seen in the capillaries and collecting ducts and the emergence of fluid from the excretory pores occurs regularly. There is no reason to doubt the distal movement of fluid in the excretory system. It may be osmoregulatory but the flow of fluid undoubtedly removes nitrogenous waste products from the body.

Development of the cercariae has been studied in serial sections of infected snails. In the morula stage of cercarial embryos a cluster of somewhat larger and more deeply staining cells is visible near the middle of the embryo. These cells are germinal and eventually give rise to the gonads, the vitellaria and the reproductive ducts. But I have not been able with certainty to determine the origin of the cells that form the epithelial wall of the excretory vesicle.

Metacercaria (Figures 1 and 2)

The cercariae encyst soon after they enter the tissues of the fish. Recently formed cysts are oval, each slightly longer than the body of the cercaria, which

lies in a fluid-filled cavity. As development proceeds the larva increases in size and is bent ventrally. At first the wall is thin and flexible but it thickens, hardens and becomes very tough. The cyst shown in Figure 1 from a laboratory-reared *M. menidia*, dissected six weeks after exposure to cercariae, measures 0.28 by 0.20 mm. and the wall is 0.015 to 0.020 mm. thick. The largest cyst measured 0.37 by 0.27 mm. The cysts are not strongly enclosed by connective tissue and often drop out, especially in the pharyngeal region, as dissection is made. Released from cysts, young worms are 0.30 to 0.60 mm. in length. The specimen shown in Figure 2, fixed under coverglass pressure, is 0.62 mm. long. As development proceeds, the ocelli regress, the peristomial spines attain a length of 0.025 mm. and a width of 0.004 mm. The acetabulum and oral sucker are almost the same size, about 0.090 mm. in diameter. The pharynx, situated just anterior to the bifurcation of the digestive system, measures 0.036 to 0.043 mm. in diameter. The gonads are clearly outlined and the reproductive ducts are indicated by strands of deeply staining cells. The intestine contains small spherules, 0.005 to 0.007 mm. in diameter. The excretory vesicle is filled with minute spherules about 0.001 mm. in diameter, which render the bladder opaque and give the fluid a milky appearance when expelled.

Stephanostomum dentatum

Adult (Figures 4, 5 and 6)

Although evidence is yet lacking to prove that *Cercaria dipterocerca* is the larval stage of *Stephanostomum dentatum*, morphological agreement supports the probability that the two are conspecific.

Linton (1900) gave a brief but diagnostic description and figures of this species. The worm from *Paralichthys dentatus*, described in that paper as *Distomum* sp. and represented on Plate 40, Figures 73, 74, and 75, is probably a specimen of *S. dentatum* from which the peristomial spines had been lost. Forty years later, Linton (1940) gave a record of collections and restated the diagnostic characters of *S. dentatum*. He determined the number of peristomial spines as 54, their length as about 0.050 mm.; he reported that they are nearly uniform in diameter for the basal half of their length, then taper gradually to the tip, which is sharply pointed. He noted the frequent loss of spines from both the peristomial rows and the general body-surface. He recognized that the mass of spermatozoa in the female duct is in the initial portion of the uterus, not in a seminal vesicle. The largest measured specimens were 2.80 mm. long by 0.100 mm. wide and 2.91 mm. long by 0.77 mm. wide. Specimens deposited in the Helminthological Collection of the U. S. National Museum are mounted on two slides which bear the numbers 8199 and 8200. Slide No. 8199 bears twelve specimens collected October 6, 1911, by Vinal Edwards. The largest worm is 2.31 mm. long and 0.81 mm. wide. The entire surface of the body is spined and the circumoral spines (shown in Figure 5) are clearly 54 in number. This specimen is designated as type of *S. dentatum*. The smallest worm, shown in Figure 4, is 0.70 mm. long; another, 0.94 mm. long, has no eggs in the uterus, and one 1.06 mm. long contains several eggs. The specimen shown by Linton (1900; Plate 39, Fig. 64) contains eggs. According to the legend the figure is × 100, and since it measures only 80 mm., the

worm was less than 1.00 mm. long. The largest specimen collected during the present investigation, fixed under coverglass pressure and shown in Figure 6, measures 2.79 mm. in length. It is clear that the worms become gravid at a length of about 1.00 mm. and most of them when stained and mounted do not exceed 2.00 mm. in length.

The single specimen on Slide 8200, Helminth. Coll., collected September 5, 1919, presumably by Professor Linton, and figured in his (1940) paper (Plate 3, Fig. 25) as a representative of *S. dentatum*, measures 3.50 mm. in length and is shown in Figure 8 and 8a. All the spines, except for a few peristomial ones, have been lost and vestiges of the eye-spots remain. The sockets of certain of the peristomial spines are visible and the few spines which remain are broad, flat (Fig. 8a), and taper abruptly to sharp points. They are 0.045 to 0.050 mm. in length. While the over-all morphology is that of *Stephanostomum*, the vestiges of ocellar pigment indicate that the specimen is young, and two features, its size and the shape of the peristomial spines, denote that it is not *S. dentatum*. It probably represents an as yet unrecognized and unnamed species.

Linton (1905) reported *S. dentatum* from seven host species: *Coryphaena equisetis, Lophopsetta maculata, Micropogon undulatus, Paralichthys albiguttus, Paralichthys dentatus, Pomatomus saltatrix*, and *Rachycentron canadus*, at Beaufort, North Carolina. Manter (1931) listed *S. dentatum* as a common parasite of *P. dentatus* at Beaufort, but he (1947) reported that some of the specimens of Linton were not suitable for identification and that *S. dentatum* seems characteristically to be a parasite of flounders. In this paper, Manter (1947) reported *S. dentatum* from the marine fishes, *Epinepheles adscencionis, Epinepheles morio* and *Mycteroperca venenosa* at Tortugas, Florida. But the material consisted of only three specimens and a fragment that lacked both tips. He noted that in the specimens from Florida the gonads were separated and the intervals between them contained vitelline follicles, whereas in the specimens from Beaufort the gonads were close together and not separated by vitellaria. Since this appeared to be a constant character, he suggested that additional material might justify a new species for the Tortugas specimens. In the worm from *E. adscencionis*, shown in his Figure 61, the gonads are smaller and situated more posteriad than in specimens of *S. dentatum* (compare with Figure 6 in this paper). In the Florida material, the separateness of the gonads may be correlated with their smaller size, which permitted extension of the vitellaria into areas between them. The smaller size of the gonads may be due to genetic factors or to development in unusual hosts, or indeed, the worms may not be normal parasites of the fishes in which they were found; instead, they may have been ingested in other hosts. Digenetic trematodes of fishes may persist for some time in the digestive tract of predators and such a possibility should be considered when dealing with instances where the worms are irregular in occurrence or few in number.

Certain morphological details may be added to earlier descriptions. In unpressed specimens the body, especially the postacetabular region, is almost circular in cross-section. The prepharyngeal region, when elongated, is slender, which gives the oral sucker a funnel-like or bell-shaped appearance. In young specimens, before the vitelline follicles develop, the connections between the intestinal ceca and the excretory vesicle to form the uroproct are easily seen. The peristomial

spines (Fig. 5) are slightly longer than 0.050 mm. The gonads are almost if not quite contiguous and frequently overlap, the ovary is situated slightly on the right side, the anterior testis sometimes slightly left. The seminal vesicle is followed by a prostatic portion of the male duct and both the cirrus and metraterm bear minute spines. The cirrus is protrusible and in one specimen measures 0.19 mm. long and 0.08 mm. wide.

Discussion

The tendency for members of *Stephanostomum* to lose cuticular spines has led to confusion and taxonomic difficulties as noted by Pratt (1916), Manter (1934) and other authors. Attempts to correlate particular larval and adult stages have not been fruitful, although the taxonomic problems, identity and number of valid species, and systematic position of the genus will not be finally resolved until life-cycle data are available.

Differences in the accounts of Martin (1939) and Wolfgang (1954, 1955a) on the life-histories of *S. tenue* and of *S. baccatum*, respectively, are perplexing. According to Martin, the cercaria of *S. tenue* has a simple tail with a sticky tip, a stenostomate excretory pattern and a flame-cell formula of 2 (3 + 3 + 3 + 3 + 3 + 3 + 3). According to Wolfgang, the cercaria of *S. baccatum* lacks a stylet and peristomial spines, has a simple tail with a sticky tip, a mesostomate excretory pattern with the pore at the tip of the tail, but the flame-cell pattern was not worked out. Wolfgang noted that the cercaria of *S. baccatum* is distinctly different from that of *S. tenue*. But his observation that the excretory vesicle discharges through a duct which traverses the tail and opens at the tip is so unusual that it can be accepted only after confirmation. Moreover, his description of a mesostomate excretory pattern in the cercaria and a stenostomate arrangement in the metacercaria would necessitate a major alteration in the collecting tubules and suggests that the cercaria and metacercaria belong to different species.

Cercaria dipterocerca differs from the cercaria described by Martin as the larva of *S. tenue* in several important features. It lacks a stylet, has lateral and dorsoventral cuticular fins on the tail, and the flame-cell formula is simpler. 2 (3 + 3 + 3). These differences are far greater than ordinarily encountered between cercariae of species in a single genus and present as yet unresolved problems.

The genus *Stephanostomum* has been assigned to the family Acanthocolpidae but the relationships of that family are uncertain. Most authors have included the Acanthocolpidae in the Allocreadioidea. La Rue (1957) presented a classification of the digenetic trematodes based on life-history data and designed to show genetic relationships. The system was founded primarily on the formation of the excretory bladder, supplemented by other morphological and bionomic data. The Digenea were divided into the Anepitheliocystidia and the Epitheliocystidia. The first group comprised three orders; Strigeatoidea, Echinostomida, and Renicolida, in which the excretory vesicle is thin, membranous, not epithelial at any stage, and in which the cercariae have forked or single tails and the excretory ducts extend into the tails of developing cercariae. In the second group, the excretory vesicle has a primary membranous wall which is replaced by an epithelium derived from a mesodermal cell-mass. It comprises two orders: the Plagiorchiida and

the Opisthorchiida. These groups were distinguished by the location of the primary excretory pores, which in the Plagiorchiida are at the furrow between body and tail and in the Opisthorchiida on the lateral margins of the tail, near its base. The order Plagiorchiida contains two superfamilies: the Plagiorchioidea Dollfus, 1930 and the Allocreadioidea Nicoll, 1934. As noted, *Stephanostomum* has been accepted as a member of the Acanthocolpidae in the Allocreadioidea. But in *Stephanostomum* the excretory ducts extend into the tail of the cercaria, which would exclude the genus from the Allocreadioidea and require its transfer to the Opisthorchioidea. Such a disposition would require that the genus be removed from the Acanthocolpidae or the transfer of the entire family. Indeed, such an allocation has been suggested; at the annual Midwest Conference of Parasitologists, Peters reported on the development of the excretory system in an acanthocolpid cercaria and in the mimeographed abstract of the paper stated, "Thus, the Acanthocolpidae must be excluded from the Allocreadioidea and may be closer to the Echinostomatioidea, or possibly to the Opisthorchioidea." At the same meeting, Cable, Peters and Berger discussed the affinities of the Acanthocolpidae and noting substantial agreement with members of the family Campulidae, suggested possible genetic and taxonomic relationship, even inclusion in the same family. Pande (1960) erected a new genus *Brijicola* to contain a new species, *B. caballeroi*, with morphological similarities to a number of genera at present assigned to such different families as Acanthocolpidae Lühe, 1909; Acanthostomatidae Poche, 1926 emend. Nicoll, 1935; Echinostomatidae Looss, 1902 emend. Poche, 1926; Maseniidae Yamaguti, 1953; Heterophyidae Odhner, 1914; and Plagiorchiidae Lühe, 1901 emend. Ward, 1917. The genus was assigned to the Acanthocolpidae with the comment that the present concept of the family remains greatly confused and unsatisfactory.

Summary

The morphology, composition and distribution of the genus *Stephanostomum* are considered. Taxonomic uncertainties and difficulties are discussed. It is postulated that the difficulties can be resolved only with knowledge of life-cycles, larval forms, and developmental stages. Previous accounts of life-histories in the genus are reviewed. The cercariae described by Miller and Northup (1926) from *Nassa obsoleta* at Woods Hole are listed and their life-cycles are traced. *Cercaria dipterocerca* Miller and Northup, 1926 penetrated and encysted in laboratory-reared specimens of *Menidia menidia*. They continued their development and the mature metacercariae manifest such precise agreement in number and shape of peristomial spines and in general morphology with juvenile and mature specimens of *Stephanostomum dentatum* (Linton, 1900) Manter, 1931, that *C. dipterocerca* may be the larval stage of *S. dentatum*. *Cercaria dipterocerca* is very different, however, from the cercariae described by Martin (1939) and Wolfgang (1954, 1955a) as the larvae of *Stephanostomum tenue* and *Stephanostomum baccatum*, respectively. The larvae described by Martin and Wolfgang differ markedly from one another. These differences are far greater than ordinarily encountered between species of a single genus and present as yet unresolved problems. The systematic position of *Stephanostomum* and the family to which it belongs are equivocal. If the genus is a member of the Acanthocolpidae, and if La Rue's

system is valid, the family must be transferred from the Allocreadioidea to the Opisthorchioidea.

LITERATURE CITED

BRAVO, MARGARITA H., 1956. Trematodos de peces marinos de aguas mexicanus. XI. Estudio de 17 Digeneos de las Costas del Pacifico, incluyendo seis especies nuevas y un genero nuevos. *Anal. Instit. Biol., Mexico,* **27**: 245–277.

CABALLERO, Y C., E., 1952. Revisión de los géneros y especies que integran la familia Acanthocolpidae Lühe, 1909. *Rev. Med. Vet. y Parasitol.,* **11**: 1–231.

CABLE, R. M., L. E. PETERS AND JEAN B. BERGER, 1960. The affinities of the trematode families Acanthocolpidae and Campulidae. Mimeographed abstract of paper presented at the annual Midwest Conference of Parasitologists, June, 1960.

DANIELSSEN, D. C., AND J. KOREN, 1880. Miscellaneous. *Ann. Mag. Nat. Hist., ser. 5,* **6**: 462–465.

DUJARDIN, F., 1845. Histoire Naturelle des Helminthes ou Vers Intestinaux. 654 pp., Paris.

FILIPPI, F., 1855. Deuxième Mémoire Pour Servir à l'Histoire Génétique des Trématodes. 26 pp., Turin.

JOHNSTONE, J., 1905. Internal parasites and diseased conditions of fishes. *Trans. Liverpool Biol. Soc.,* **19**: 278–300.

KRØYER, H., 1838–1840. Danmark's Fiske. 616 pp., Kjobenhavn.

LA RUE, G. R., 1957. The classification of digenetic Trematoda: a review and a new system. *Experiment. Parasitol.,* **6**: 306–344.

LEBOUR, MARIE V., 1908. Fish trematodes of the Northumberland coast. Rep. Northumberland Fish Comm. for 1907, pp. 23–67.

LEIDY, J., 1891. Notices of Entozoa. *Proc. Acad. Nat. Sci., Philadelphia,* **42**: 410–418.

LENZ, 1794. (cited after Neave, *Nomenclator Zoologicus*). N. Allg. dtsch Bibl., 10 (2), Intelligenzbl., 28, 256 (n.n.); in Joerdens, Ent. Helminth. Menschl. Körpers, 2, 29.—Dipt. (Larva).

LINTON, E., 1898. Notes on trematode parasites of fishes. *Proc. U. S. Nat. Mus.,* **20**: 507–548.

LINTON, E., 1900. Fish parasites collected at Woods Hole in 1898. Bull. U. S. Fish Comm. for 1899, pp. 267–304.

LINTON, E., 1905. Parasites of fishes of Beaufort, North Carolina. *Bull. U. S. Bur. Fisheries for 1904,* **24**: 321–428.

LINTON, E., 1910. Helminth fauna of the Dry Tortugas. II. Trematodes. Publ. No. 133, Carnegie Instit. Wash., pp. 11–98.

LINTON, E., 1915. Note on trematode sporocysts and cercariae in marine mollusks of the Woods Hole region. *Biol. Bull.,* **28**: 198–209.

LINTON, E., 1940. Trematodes from fishes mainly from the Woods Hole region, Massachusetts. *Proc. U. S. Nat. Mus.,* **88**: 1–172.

LOOSS, A., 1899. Weitere Beiträge zur Kenntnis der Trematodenfauna Aegyptens, zugleich Versuch einer natürlichen Gliederung des Genus *Distoma* Retzius. *Zool. Jahrb., System.,* **12**: 521–784.

LOOSS, A., 1900. Nachträgliche Bemerkungen zu den Namen der von mir vorgeschlagenen Distomiden gattungen. *Zool. Anz.,* **23**: 601–608.

LÜHE, M., 1906. Report on the trematode parasites from marine fishes of Ceylon. Rep. Govt. Ceylon Pearl Oyster Fish. Gulf Manaar (Herdman); Part 5, pp., 97–108.

MCFARLANE, S. H., 1934. *Stephanostomum casum* (Linton) a trematode possessing a uroproct. *Trans. Amer. Micros. Soc.,* **53**: 172–173.

MANTER, H. W., 1926. Some North American fish trematodes. *Illinois Biol. Monogr.,* **10**(2): 1–138.

MANTER, H. W., 1931. Some digenetic trematodes of marine fishes of Beaufort, North Carolina. *Parasitol.,* **23**: 396–411.

MANTER, H. W., 1934. Some digenetic trematodes from deep-water fishes of Tortugas, Florida. Pap. Tortugas Lab., No. 28. Publ. No. 435, Carnegie Instit. Wash., 257–345.

MANTER, H. W., 1940. Digenetic trematodes from the Galapagos Islands and neighboring Pacific. *Allan Hancock Pacif. Exped.,* **2**(14): 329–497.

MANTER, H. W., 1947. The digenetic trematodes of marine fishes of Tortugas, Florida. *Amer. Midl. Naturalist,* **38**: 257–416.

MANTER, H. W., AND H. J. VAN CLEAVE, 1951. Some digenetic trematodes, including eight new species, from marine fishes of La Jolla, California. *Proc. U. S. Nat. Mus.*, **101**: 315–340.

MARTIN, W. E., 1938. Studies on trematodes of Woods Hole: the life cycle of *Lepocreadium setiferoides* (Miller and Northup), Allocreadiidae, and the description of *Cercaria cumingiae* n. sp. *Biol. Bull.*, **75**: 463–474.

MARTIN, W. E., 1939. Studies on the trematodes of Woods Hole. II. The life cycle of *Stephanostomum tenue* (Linton). *Biol. Bull.*, **77**: 65–73.

MILLER, H. M., JR., AND FLORA E. NORTHUP, 1926. The seasonal infestation of *Nassa obsoleta* (Say) with larval trematodes. *Biol. Bull.*, **50**: 490–509.

MONTGOMERY, W. R., 1957. Studies on digenetic trematodes from marine fishes of La Jolla, California. *Trans. Amer. Micros. Soc.*, **76**: 13–36.

MOULINIÉ, J., 1856. De la reproduction chez les trématodes endo-parasites. *Mém. Instit. Nat. Genevois*, **3**: 7–278.

NICOLL, W., 1907. A contribution towards a knowledge of the Entozoa of British marine fishes. Pt. 1. *Ann. Mag. Nat. Hist., ser. 7*, **19**: 66–94.

NICOLL, W., 1910. On the Entozoa of fishes from the Firth of Clyde. *Parasitol.*, **3**: 322–359.

NICOLL, W., AND W. SMALL, 1909. Notes on larval trematodes. *Ann. Mag. Nat. Hist., ser. 8*, **3**: 237–246.

OLSSON, P., 1867–1868. Entozoa, iakttagnahaos skandinaviska hafsfiskar. 1. Platyhelminthes. *Lunds. Arssk. Math. Naturv.-Vetensk*, **4**: 1–64.

PANDE, B. P., 1960. On *Brijicola caballeroi*, n.g., n. sp., a digenetic trematode from the little egret. Libro homenaje al Dr. Eduardo Caballero y Caballero, pp. 227–232.

PENNER, L. R., 1953. The red-breasted merganser as a natural avian host of the causative agent of clam-diggers' itch. *J. Parasit.*, **39**(Suppl.): 20.

PÉREZ VIGUERAS, I., 1942. Notas helmintológicas, *Monorchistephanostomum gracile* n.g., n. sp. (Trematoda: Acanthocolpidae), parásito de *Sphyraena barracuda* (Pisces). Rev. Univ. Habana (Nos. 40–42), pp. 193–223.

PETERS, L. E., 1960. Development of the excretory system in four species of cercaria and its bearing on the allocreadioid problem. Mimeographed abstract of paper presented at the annual Midwest Conference of Parasitologists, June, 1960.

POCHE, F., 1926. Das System der Platodaria. *Arch. Naturg., Abt. A.*, **91**: 1–458.

PRATT, H. S., 1916. The trematode genus *Stephanostomum* Looss in the Gulf of Mexico. *Parasitol.*, **8**: 229–238.

PRICE, E. W., 1929. A synopsis of the trematode family Schistosomidae with descriptions of new genera and species. *Proc. U. S. Nat. Mus.*, **75** (18): 1–39.

SOGANDARES-BERNAL, F., AND R. F. HUTTON, 1959a. Studies on helminth parasites from the coast of Florida. III. Digenetic trematodes of marine fishes from Tampa and Boca Ciega Bays. *J. Parasitol.*, **45**: 337–346.

SOGANDARES-BERNAL, F., AND R. F. HUTTON, 1959b. Studies on helminth parasites from the coast of Florida. IV. Digenetic trematodes of marine fishes of Tampa, Boca Ciega Bays, and the Gulf of Mexico. *J. Florida Acad. Sci.*, **21**: 259–273.

STAFFORD, J., 1904. Trematodes from Canadian Fishes. *Zool. Anz.*, **27**: 481–495.

STOSSICH, M., 1883. Brani di elmintologia tergestina. *Ser. 1, Boll. Soc. Adriat. Sci. nat. Trieste*, **8**: 111–121.

STUNKARD, H. W., 1938a. The morphology and life-cycle of the trematode *Himasthla quissetensis* (Miller and Northup, 1926). *Biol. Bull.*, **75**: 145–164.

STUNKARD, H. W., 1938b. *Distomum lasium* Leidy, 1891 (syn. *Cercariaeum lintoni* Miller and Northup, 1926), the larval stage of *Zoogonus rebellus* (Olsson, 1868) (syn. *Z. mirus* Looss, 1901). *Biol. Bull.*, **75**: 308–334.

STUNKARD, H. W., 1941. Specificity and host-relations in the trematode genus *Zoögonus*. *Biol. Bull.*, **81**: 205–214.

STUNKARD, H. W., AND M. C. HINCHLIFFE, 1952. The morphology and life-history of *Microbilharzia variglandis* (Miller and Northup, 1926) Stunkard and Hinchliffe, 1951, avian blood-flukes whose larvae cause "swimmer's itch" of ocean beaches. *J. Parasitol.*, **38**: 248–265.

WARD, H. B., 1938. On the genus *Deropristis* and the Acanthocolpidae. Livro Jubilar Prof. Lauro P. Travassos. Rio de Janeiro, pp. 509–521.

WOLFGANG, R. W., 1954. Studies of the trematode *Stephanostomum baccatum* (Nicoll, 1907): II. Biology with special reference to the stages affecting the winter flounder. *J. Fish. Board, Canada*, **11**: 963–987.

WOLFGANG, R. W., 1955a. Studies on the trematode *Stephanostomum baccatum* (Nicoll, 1907. III. Its life cycle. *Canad. J. Zool.*, **33**: 113–128.

WOLFGANG, R. W., 1955b. Studies on the trematode *Stephanostomum baccatum* (Nicoll, 1907). IV. The variation of the adult morphology and the taxonomy of the genus. *Canad. J. Zool.*, **33**: 129–142.

YAMAGUTI, S., 1934. Studies on the helminth fauna of Japan. Part 2. Trematodes of fishes. I. *Jap. J. Zool.*, **5**: 249–541.

YAMAGUTI, S., 1937. Studies on the helminth fauna of Japan. 20. Larval trematodes from marine fishes. *Jap. J. Zool.*, **7**: 491–499.

YAMAGUTI, S., 1958. *Systema Helminthum*, vol. 1, Digenetic Trematodes. 1575 pp. Interscience Publishers, Inc. New York.

THE PARTITIONING OF BODY WATER IN OSTEICHTHYES: PHYLOGENETIC AND ECOLOGICAL IMPLICATIONS IN AQUATIC VERTEBRATES[1]

THOMAS B. THORSON

Department of Zoology, University of Nebraska, Lincoln 8, Nebraska

Great strides have been made in recent years in understanding the fluid medium in which metabolic processes occur. Investigations have been largely confined to qualitative studies of the constituents of the body fluids and to the relationship of qualitative shifts to water balance or imbalance. Although water itself is the fundamental substance involved in fluid balance, it is a curious fact that, until recently, there had apparently been no attempt made to determine the apportionment of the total body water among the fluid compartments of vertebrates, except in man and a few other mammals.

Reichert and Brown (1909) and more recently Florkin (1949), among others, have ascribed both phylogenetic and ecological significance to qualitative features of body fluids. It was thought that a comparative, quantitative study of vertebrate fluid compartments might demonstrate patterns which would likewise have phylogenetic and/or ecological implications. Data have been published on the Chondrichthyes (Thorson, 1958) and on Agnatha (Thorson, 1959), and this paper presents findings on the third aquatic vertebrate class, Osteichthyes, the bony fishes.

Only six papers known to the writer have previously reported measurements of blood volume in bony fishes. These are listed in Table III, which will be treated later. Only Prosser and Weinstein (1950) have attempted measurement of the extracellular fluid volume (NaSCN space) of fish, in a single species; but no attempt has previously been made at a complete quantitative analysis of the major fluid compartments of Osteichthyes.

To the following people grateful acknowledgement is extended for the use of laboratory and collecting facilities and providing experimental animals: William H. Sutcliffe, Jr., Director, and Brunell Spurling, Collector, Bermuda Biological Station; F. G. Walton Smith, Director, Hilary B. Moore, Assistant Director, and Charles E. Lane, Research Associate, University of Miami Marine Laboratory; Craig Phillips, Curator, and Capt. William Gray, Collector, Miami Seaquarium; W. F. Rolleston, General Manager, F. G. Wood, Jr., Curator, Clifford Townsend, Assistant Curator, and Ronnie Capo, Collector, Marineland Research Laboratory; M. O. Steen, Director, Glen R. Foster, Chief of Fisheries Division, and Gerhard Lenz, Superintendent of Gretna Hatchery, Nebraska Game, Forestation and Parks

[1] Contribution No. 279 from the Bermuda Biological Station, and Studies from the Department of Zoology, University of Nebraska, No. 328. This investigation was supported in 1956 by National Science Foundation grant No. 1154 and by a grant-in-aid from the National Science Foundation through the Bermuda Biological Station. In 1957 and 1958 it was supported by United States Public Health Service grant No. H-3134, and by the University Research Council of the University of Nebraska.

TABLE I

Summary of experimental data. The mean is followed by the number of experimental animals (in parentheses). The range of measurements appears in parentheses under the mean. Standard deviation is given for plasma, extracellular fluid and total body water

	Fresh-water Chondrostei		Fresh-water Holostei		Fresh-water Teleostei		
	Lake sturgeon	Paddlefish	Bowfin	Shortnose gar	Common sucker	Carp	Bigmouth buffalofish
Weight (gm.)	3058 (8) (2275–4530)	4679 (5) (3740–5910)	1963 (6) (1020–3265)	1185 (7) (855–1730)	617 (2) (580–655)	2412 (7) (1585–3190)	3395 (8) (1980–5440)
Pulse (beats/min.)	49 (8) (44–52)	22 (5) (16–28)	20 (6) (14–28)	19 (6) (14–24)	55 (2) (47–64)	28 (6) (18–39)	38 (8) (19–70)
Respiration (per min.)	53 (8) (40–72)	17 (5) (10–26)	14 (6) (9–20)	28 (5) (24–36)	47 (2) (45–50)	27 (3) (19–38)	22 (4) (20–24)
Hematocrit (per cent cells)	22 (8) (19–29)	30 (5) (24–37)	32 (6) (32–34)	42 (7) (33–50)	39 (2) (39–40)	33 (7) (23–44)	29 (8) (18–40)
Spec. grav., plasma	1.016 (3) (all 1.016)	1.017 (3) (1.016–1.018)	1.018 (3) (1.0175–1.0185)	1.019 (3) (1.016–1.021)	1.016 (3) (1.015–1.017)	1.019 (3) (1.018–1.020)	1.016 (3) (all 1.016)
Spec. grav., blood	1.036 (3) (1.033–1.040)	1.040 (3) (1.039–1.041)	1.045 (3) (1.044–1.047)	1.051 (3) (1.050–1.052)	1.041 (3) (1.040–1.042)	1.040 (3) (1.039–1.0405)	1.042 (3) (1.041–1.043)
Plasma volume (T-1824 space)*	2.8 (8) (2.2–3.9) s.d., .50	2.2 (5) (1.8–2.3) s.d., .21	2.2 (6) (1.9–3.2) s.d., .46	2.1 (7) (1.6–2.6) s.d., .38	1.2 (2) (1.0–1.5) s.d., .25	1.8 (7) (1.4–2.2) s.d., .27	1.9 (8) (1.3–3.3) s.d., .61
Blood volume*	3.7 (8) (2.8–4.9)	3.0 (5) (2.4–3.6)	3.4 (6) (2.9–5.0)	3.8 (7) (3.0–5.2)	2.2 (2) (1.8–2.7)	3.0 (7) (2.4–3.5)	2.8 (8) (1.8–4.1)
Extracellular fluid (sucrose space)*	20.1 (8) (15.4–27.5) s.d., 3.91	15.6 (5) (14.1–16.9) s.d., .95	18.9 (6) (14.6–25.6) s.d., 3.31	13.6 (7) (12.5–14.8) s.d., .86	12.2 (2) (11.8–12.7) s.d., .45	15.5 (7) (12.0–22.1) s.d., 3.34	13.2 (8) (10.9–15.6) s.d., 1.27
Interstitial fluid (sucrose space minus plasma)*	17.3	13.4	16.7	11.5	11.0	13.7	11.3
Total body water*	72.7 (8) (71.5–74.0) s.d., .70	74.0 (5) (71.3–77.4) s.d., 2.07	74.5 (6) (72.6–76.4) s.d., 1.18	66.7 (7) (64.5–70.7) s.d., 1.85	74.4 (2) (74.1–74.8) s.d., .35	71.4 (7) (70.1–73.1) s.d., 1.19	70.6 (8) (64.1–76.0) s.d., 3.45
Intracellular fluid (total water minus sucrose space)*	52.6	58.4	55.6	53.1	62.2	55.9	57.4

* All fluid volumes expressed as per cent of body weight.

TABLE I—*Continued*

	Marine Teleostei						
	Tiger rockfish	Nassau grouper	Red snapper	Gray snapper	Green moray	Great barracuda	Rainbow parrotfish
Weight (gm.)	5885 (1)	1270 (2) (930–1610)	3765 (2) (3130–4400)	3711 (6) (1900–4680)	4062 (6) (3050–4815)	2204 (11) (1430–4575)	4607 (19) (1650–6830)
Pulse (beats/min.)	51 (1)	52 (1)	58 (2) (48–68)	54 (3) (44–66)	70 (5) (60–88)	68 (11) (32–98)	48 (16) (30–98)
Respiration (per min.)	—	—	—	41 (4) (30–48)	22 (3) (18–28)	41 (7) (30–54)	40 (14) (22–64)
Hematocrit (per cent cells)	28 (1)	28 (2) (28–29)	36 (2)	35 (6) (28–40)	26 (6) (24–28)	31 (11) (25–36)	30 (27) (20–40)
Spec. grav., plasma	Used ave. (1.017)	Used ave. (1.017)	Used ave. (1.017)	Used ave. (1.017)	Used ave. (1.017)	Used ave. (1.017)	Used ave. (1.017)
Spec. grav., blood	Used ave. (1.042)	Used ave. (1.042)	Used ave. (1.042)	Used ave. (1.042)	Used ave. (1.042)	Used ave. (1.042)	Used ave. (1.042)
Plasma volume (T-1824 space)*	2.3 (1)	1.8 (2) (1.6–2.1) s.d., .25	1.3 (2) (1.3–1.4) s.d., .05	1.3 (6) (1.2–1.5) s.d., .10	1.6 (6) (0.7–2.3) s.d., .63	1.9 (10) (1.5–2.6) s.d., .30	2.4 (16) (1.9–3.2) s.d., .42
Blood volume*	3.3 (1)	2.6 (2) (2.3–3.0)	2.2 (2)	2.0 (6) (1.9–2.5)	2.2 (6) (1.0–3.0)	2.8 (10) (2.3–3.7)	3.6 (16) (2.5–4.7)
Extracellular fluid (sucrose space)*	12.5 (1)	14.5 (2) (13.1–16.0) s.d., 1.45	14.0 (2)	14.0 (6) (12.2–15.3) s.d., .97	15.8 (6) (12.2–19.4) s.d., 2.67	15.9 (8) (11.6–19.1) s.d., 2.07	16.6 (8) (14.3–18.9) s.d., 1.60
Interstitial fluid (sucrose space minus plasma)*	10.2	12.7	12.7	12.7	14.2	14.0	14.2
Total body water*	71.1 (1)	71.7 (2) (70.4–73.1) s.d., 1.35	71.3 (2) (71.2–71.5) s.d., .15	72.3 (6) (70.5–74.0) s.d., 1.02	63.7 (6) (56.1–71.8) s.d., 5.92	70.6 (9) (68.1–73.3) s.d., 1.94	73.1 (14) (71.2–75.0) s.d., 1.20
Intracellular fluid (total water minus sucrose space)*	58.6	57.2	57.3	58.3	47.9	54.7	56.5

TABLE II

Experimental data summarized by taxonomic groups and compared with data for Agnatha and Chondrichthyes. Mean is followed by number of experimental animals in parentheses. Standard deviation given for plasma, extracellular fluid and total body water. Ranges can be determined from Table I

	Agnatha (*Petromyzon marinus*)	Chondrichthyes (summary)	Osteichthyes (summary)	Fresh-water Chondrostei	Fresh-water Holostei	Fresh-water Teleostei	Marine Teleostei
Weight (gm.)	190 (12)	3012 (65)	3195 (90)	3681 (13)	1544 (13)	2664 (17)	3710 (47)
Pulse (per min.)	31 (12)	24.5 (39)	44.5 (80)	39 (13)	20 (12)	37 (16)	57 (39)
Respiration (per min.)	122 (12)	—	35.3 (61)	47 (13)	20 (11)	29 (9)	38 (28)
Hematocrit (per cent cells)	33 (12)	18.3 (52)	30.7 (98)	25 (13)	38 (13)	32 (17)	30 (55)
Spec. grav., plasma	1.018 (12)	—	1.017 (21)	1.0165 (6)	1.0185 (6)	1.017 (9)	—
Spec. grav., blood	1.040 (12)	—	1.042 (21)	1.038 (6)	1.048 (6)	1.041 (9)	—
Plasma volume (T-1824 space)*	5.5 (12) s.d., .32	5.4 (44) s.d., 1.3	2.0 (86) s.d., .41	2.5 (13) s.d., .41	2.1 (13) s.d., .42	1.8 (17) s.d., .46	1.9 (43) s.d., .38
Blood volume*	8.5 (12)	6.6 (44)	3.0 (86)	3.5 (13)	3.6 (13)	2.8 (17)	2.9 (43)
Extracellular fluid (sucrose space)*	23.9 (12) s.d., .79	21.2 (3) s.d., 2.38	15.7 (76) s.d., 2.28	18.4 (13) s.d., 3.12	16.0 (13) s.d., 2.34	14.0 (17) s.d., 2.31	15.4 (33) s.d., 1.80
Interstitial fluid (sucrose space minus plasma)*	18.4 (12)	15.8	13.7	15.9	13.9	12.2	13.5
Total body water*	75.6 (12) s.d., .51	74.8 (42) s.d., 1.6	71.2 (83) s.d., 2.29	73.2 (13) s.d., 1.40	70.3 (13) s.d., 1.58	71.4 (17) s.d., 2.47	70.8 (40) s.d., 2.62
Intracellular fluid (total water minus sucrose space)*	51.7	53.6	55.5	54.8	54.3	57.4	55.4

* All fluid volumes expressed as per cent of body weight.

TABLE III

Blood volumes of Osteichthyes reported in the literature

Author	Species	Method	No. of specimens	Per cent body weight
Welcker (1858)	*Cyprinus tinea*	Direct (Hb)	1	1.87
Welcker (1858)	*Perca fluviatilis*	Direct (Hb)	1	1.07
Derrickson and Amberson (1934)	*Tautoga onitis*	Direct (Hb)	3	1.5
Prosser and Weinstein (1950)	*Ictalurus natalis*	T-1824	6	1.77
Martin (1950)	*Ophiodon elongatus*	T-1824	8	2.8
Martin (1950)	*Ophiodon elongatus*	Vital Red	1	1.9
Martin (1950)	*Sebastodes sp.*	Vital Red	1	2.8
Martin (1950)	Cottidae (sculpin)	Vital Red	3	2.3
Lennon (1954)	*Catostomus commersoni*	Tail severance (bleeding)	23	1.5
Schiffman and Fromm (1959)	*Salmo gairdneri*	T-1824	10	2.25

Commission; James T. Shields, Superintendent of Fisheries, South Dakota Department of Game, Fish and Parks; E. C. Saeugling, U. S. Fish and Wildlife Service Hatchery Manager, Guttenberg, Iowa; K. H. Loftus, Head, Fisheries Section, and W. J. Christie, Biologist, Division of Research, Ontario Department of Lands and Forests.

Materials and Methods

Of the marine fish employed (all Teleostei), the great barracuda, *Sphyraena barracuda*, rainbow parrotfish, *Pseudoscarus guacamaia*, tiger rockfish, *Mycteroperca tigris*, and one gray snapper, *Lutianus griseus*, were collected from the waters surrounding the islands of Bermuda. The remainder of the gray snappers and the red snappers, *Lutianus campechanus*, were taken off the coast of Florida in the general vicinity of St. Augustine. The Nassau grouper, *Epinephelus striatus*, and green moray, *Gymnothorax funebris*, were collected off the Florida coast near the Miami area.

Of the fresh-water fish, all the teleosts were taken from Nebraska lakes or streams: bigmouth buffalofish, *Ictiobus cyprinellus*, carp, *Cyprinus carpio*, and common white sucker, *Catostomus commersoni*. Of the Holostei (fresh-water forms), the shortnose gar, *Lepisosteus platostomum*, were taken in eastern Nebraska, and the bowfin, *Amia calva*, from northeastern Iowa. Of the fresh-water Chondrostei, the paddlefish, *Polyodon spathula*, were from eastern Nebraska, and the lake sturgeon, *Acipenser fulvescens*, from the St. Lawrence River in southern Ontario.

All fish were taken in nets or traps, so were undamaged, except the lake sturgeon, which were hooked by commercial fishermen and held in a large tank for several weeks before they were used.

The fish were anesthetized with approximately 50 mg./kg. aqueous pentobarbital sodium (Nembutal) injected intraperitoneally. Respiratory movements usually continued, but the gills were irrigated artificially throughout the experiments. The anesthetic required about 30–60 minutes to take full effect, and anesthesia lasted for several hours.

Plasma and extracellular fluid volumes were measured by dilution methods modified from Keith, Rowntree and Geraghty (1915). T-1824 (Evans Blue) was employed for the former, and sucrose for the latter.

After a fish was anesthetized, a cardiac puncture was made and blood was drawn into heparinized capillary tubes for hematocrit determination. A measured quantity of blood was then drawn into aqueous potassium oxalate. The syringe was doubly calibrated to receive nine parts of blood and one part oxalate. This blood was centrifuged and the plasma was used in the blank and standard dilutions employed in colorimetry, as a control on substances normally present in plasma. A solution containing 25 mg. % T-1824 and 3% sucrose was next injected into the heart in a quantity equal to that of the blood drawn. After mixing had occurred, samples of blood were withdrawn from the heart for colorimetric determination of the dilution of the injected materials. By comparison with known dilutions, the volume occupied by the dye or sucrose could readily be calculated. However, since both T-1824 and sucrose disappear slowly from the blood, a volume calculated at one instant will be smaller than one calculated later. To obtain the theoreti-

cal concentration with complete mixing but no loss from the system, several blood samples were withdrawn over a period of time and the optical density of T-1824 and sucrose extrapolated to the time of injection (Erlanger, 1921). Equilibration of the dye was rapid, and samples were drawn at 5–10-minute intervals for about 40 minutes. Extrapolation curves showed that sucrose required about an hour to be distributed thoroughly, so less frequent samples were drawn over a period of 3–5 hours for extracellular fluid measurements. All plasma for blank, standard dilutions, and tests samples was diluted 1 : 10 with isotonic saline.

Sucrose analysis was by a modification of the method of Harrison (1942). To remove the dye complexed with plasma proteins, the plasma was deproteinized by the zinc sulphate method of Somogyi (1930). Blood glucose, which would interfere with the analysis, was removed by Little's (1949) method of heating the plasma with sodium hydroxide. Color was developed by the standard diphenylamine method, and optical density was determined with a Bausch and Lomb Spectronic 20 colorimeter at 620 mμ, as it was also for T-1824.

Whole blood volume was calculated from plasma volume and hematocrit, and total body water was determined by complete desiccation of the whole animal at 105° C.

For conversion of volume measurements to per cent of body weight, specific gravity of whole blood and plasma were required. These were determined by the method described by Todd and Sanford (1943), in which a mixture of two miscible substances (chloroform, s.g. 1.489, and benzene, s.g. .879) was adjusted to suspend a drop of the test substance. The specific gravity of the mixture was then determined by a small hydrometer, suitably calibrated and corrected for temperature.

Discussion of Results

A summary of data, by species, is presented in Table I. Each mean figure is followed in parentheses by the number of experimental animals employed. Below the mean is the range of measurements. For plasma volume, sucrose space, and total body water, the three basic fluid measurements, the standard deviation is also given. All fluid compartment measurements are expressed as percentages of body weight.

The figures for species in which only one or two animals were used cannot be regarded as reliable in view of the individual variations evident in the tabulation of ranges. Nevertheless, these have been included in the table and have been used for group comparisons in Table II. In the latter table, species have been summarized into four groups, to make possible comparisons based on habitat and major taxonomic groups. To these have been added data from previous work by the author on the sea lamprey, *Petromyzon marinus*, an agnathan (Thorson, 1959), and four species of Chondrichthyes: *Squalus acanthias*, the spiny dogfish; *Raja binoculata*, the big skate; *Raja rhina*, the long-nosed skate; and *Hydrolagus collici*, the rat-fish or chimaera (Thorson, 1958).

The paddlefish and sturgeon are members of the Chondrostei, which have a prominent, persistent notochord, a cartilaginous skeleton, and a heterocercal tail. They are generally considered the most primitive of living ray-finned fishes and, of all the species used, most closely allied to the ancestral stock. The bowfin and gar belong to the Holostei, which in general exhibit a less prominent notochord, a

partially ossified skeleton, and an abbreviated heterocercal tail. They are thought to have arisen from the chondrostean line and are more advanced than the latter, but primitive in relation to the teleosts. The Teleostei include the most advanced of the fishes and are now the dominant form of life in most waters of the earth. They have a largely ossified skeleton, the least persistent notochord, and usually a homocercal tail. They are represented in this work by ten species, the buffalofish, sucker and carp from fresh water, and the remainder from salt water. Only teleosts have been used for comparison of fresh-water with salt-water forms.

Weight

Ideally, when species are compared, animals of comparable size should be used. For practical reasons concerning supply of animals, this was not possible. Martin (1950), working with *Squalus acanthias*, reported a greater blood volume in small fish than in large ones of the same species. If this phenomenon is of general application, comparative size would be a matter of great concern. Although some series of present measurements suggested the same findings as Martin's, the pattern was not consistent enough to warrant a positive statement. Species have therefore been compared regardless of size, although reservations on these grounds may be in order.

Respiratory movements

The rates of respiratory movements were determined after anesthesia and cannot be assumed to reflect accurately the respiratory activities in unanesthetized animals. Considerable diversity exists, figures ranging from 14 per minute in the bowfin to 53 in the lake sturgeon. Great diversity is evident within each of the four major groups, and no consistent pattern is discernible which can be related to taxonomic categories. Three of the primitive species have among the lowest respiratory rates (gar, bowfin and paddlefish), but the sturgeon tends to negate the conclusion that a slow respiratory rate is a primitive characteristic. The average rate of all fresh-water species, regardless of taxonomic group, is about 33, as compared with 38 for marine species. It is tempting to speculate on the ecological significance, but actually this is not a great difference in view of the great diversity within both groups. It would seem reasonable that the rate of respiratory movements would be related to metabolism, but figures are not available on metabolic rates of these species. The possibility also suggests itself that a correlation might exist between respiratory rate and general visible activity. No quantitative study was made, but general observations do not support such conclusions. It is true that the paddlefish (17 per minute) is relatively sluggish. After removal from water it scarcely struggled, and could probably be worked on without anesthesia, although this was not done. The bowfin, however (14 per minute), was far from sluggish and docile, and the sturgeon (53 per minute), although not as sluggish as the paddlefish, was far less active than most of the other species.

Pulse

The pulse rates were also taken immediately after anesthesia, so cannot with confidence be compared with rates of unanesthetized animals. The most striking

observation from the present measurements is the rather close correspondence of relative pulse rate with the rate of respiratory movements. With only minor discrepancies, a relatively high rate of heart beat is found in species with a high respiratory rate. This is also apparent when the four summarized groups are compared. However, in this case, there is an appreciable difference between the average pulse rate of marine teleosts (57 per minute) and fresh-water teleosts (37) or the average of all fresh-water species of Osteichthyes, including the primitive one (32). The higher pulse rate, perhaps together with the somewhat higher rate of respiratory movements of marine fish, is very likely related to the difference in external medium and/or to basal metabolism and its visible manifestation, relative bodily activity. These data point up strongly the need for work on metabolic rates in these species. Since the gar, bowfin and paddlefish have decidedly the lowest pulse rates of all species studied, a strong case could be made for slow pulse as a primitive characteristic, but for the sturgeon, whose pulse rate of 49 is in the range of teleost rates. Even so, the average of all the Chondrostei and Holostei is 29 (fresh-water teleosts 37, marine teleosts 57) so it might be said that a slow pulse is a tendency in the primitive fishes, even though the lake sturgeon is an exception.

Specific gravity of plasma and whole blood

Specific gravities were determined only for fresh-water species, so comparisons cannot be made between species of different external medium. No correlation is evident between specific gravity of whole blood and phylogenetic position of the three groups of fresh-water fish. The differences between plasma specific gravity values for the three groups are so small as to be virtually meaningless. No published plasma values were available for marine fish. Martin's (1950) average figure for whole blood of three ling cod (*Ophiodon elongatus*) was 1.044. For purposes of calculations in the marine species, the mean values obtained for fresh-water fish were used (1.017 for plasma; 1.042 for whole blood).

Hematocrit

The per cent of cells in the blood was determined in each animal for calculation of whole blood from plasma volume. The results are in general agreement with figures given by Martin (1950) and Prosser and Weinstein (1950), although they are far above the 7% figure given by Welcker (1858), which must have been in error. There is little difference between the hematocrit of marine teleosts (30) and fresh-water teleosts (32) or all fresh-water species (31). There is no clear correlation with the phylogenetic sequence, nor with differences in pulse rate or respiratory movements. However, the differences in hematocrit of the three groups of fresh-water species correspond, in a manner that could be expected, with the differences in specific gravity of whole blood.

Plasma and whole blood volume

Techniques of plasma and blood volume determination have been debated vigorously for many years and have been discussed most recently by Gregersen and Rawson (1959). It is recognized that the T-1824 dilution method has certain limitations, but still more serious objections have been raised to many

other methods. In light of published discussions, as well as for practical considerations, T-1824 was selected for earlier work by the writer (1958, 1959). For the purpose of reliable comparison, it has been used in this investigation and will continue to be used in the other vertebrate groups.

When a dye is introduced into the bloodstream it is distributed only in the fluid portion of the blood, and therefore calculation of the volume it occupies is a measure of the plasma rather than whole blood. Confusion on this point (see discussion in Gregersen and Rawson, 1959) by some early users of the dilution method resulted in some plasma volumes being published as blood volumes. As a part of a study of partitioning of body fluids, plasma volume is the pertinent measurement since the water in the corpuscles is not a part of the extracellular or vascular water, but is rather a part of the intracellular compartment. However, from the standpoint of the heart and general circulation, the whole blood volume is the important consideration. Measurements of both volumes are included here.

Plasma and blood volumes of bony fishes have long been known to be low in comparison with those of other vertebrates, but recorded measurements have been rather fragmentary. All known previous measurements concerned exclusively with Osteichthyes are included in Table III. Most of the earlier results are lower than mine. However, in the most extensive study (Martin, 1950), all individual figures were within the range of variation of individual teleosts in the present study (Table I), and the average figure (2.6% of body weight) of Martin's teleosts (all marine) was only slightly lower than my marine teleosts (2.9%) shown in Table II.

It was suggested by the writer (1959) that, among aquatic vertebrate classes, a relatively large plasma and blood volume might be regarded as a primitive characteristic. *Petromyzon marinus* (Agnatha, the most primitive class of vertebrates) has a plasma volume of 5.5% of the body weight, and blood volume of 8.5% (Table II). Chondrichthyes, also a relatively primitive class, but more advanced than agnathans, has virtually the same plasma volume (5.4%), but, in agreement with the lower hematocrit value, has a lower blood volume (6.6%). None of the species of Osteichthyes, the most advanced of the three aquatic vertebrate classes, had a plasma volume of more than 2.8% (lake sturgeon) or a blood volume of more than 3.8% (shortnose gar). The case for the phylogenetic significance of vascular volume is supported by small differences in plasma volume in the three groups of fresh-water Osteichthyes. The Chondrostei, the most primitive, averaged 2.5%, the intermediate Holostei 2.1%, and the most advanced Teleostei 1.8%. The same is evident for blood volumes except that the higher hematocrit value of the Holostei increases their blood volume (3.6%) to slightly more than that of the Chondrostei (3.5%). All four of the primitive species consistently equal or exceed all species of teleosts in plasma and blood volume, except the tiger rockfish, whose single specimen is by itself virtually meaningless, and the rainbow parrotfish. A satisfactory explanation of the latter species' relatively high plasma volume (2.4%) and blood volume (3.6%) has not been found. Sufficient specimens (16) were employed to obtain reliable results; the species cannot be considered as primitive among the teleosts on morphological grounds; its external medium is not different from that of the other marine species; comparative metabolic rates are not known, and relative activity

was not determined; and Martin's (1950) size:blood volume relationship cannot account for the "discrepancy," since these fish were among the largest employed.

A comparison of fresh-water teleosts with marine teleosts reveals a striking similarity in both plasma and blood volumes, only 0.1% separating the two groups in both cases. Considering the radically different osmotic environment of the two groups and the great divergence in physiology of water balance, this is a remarkable example of phylogenetic homeostasis.

The difference between the two groups is so slight as perhaps to be meaningless, but the fact that the figures for the marine species are higher than those for fresh-water species should be kept in mind when the sucrose space is considered later.

Extracellular fluid volume (sucrose space)

The closest approximation to the extracellular fluid volume attainable with available methods is the calculation of the volume occupied by a substance which, when injected into the blood stream, will filter readily from the capillaries into the intercellular spaces. The substance must not be metabolized or excreted

TABLE IV

Comparison of inulin, raffinose and sucrose spaces of Pseudoscarus guacamaia

	Vol. (per cent body wt.)	Range	Aver. wt. (grams)	No. of specimens
Inulin	11.4	9.2–14.5	5451	8
Raffinose	14.4	12.7–16.4	4096	4
Sucrose	16.6	14.3–18.9	4696	8

rapidly, and must not be extensively taken up by the cells. It should also penetrate the minor fluid compartments, such as coelomic, cerebrospinal, and ocular fluids, which are likewise extracellular. A substance which satisfies all of these demands has not yet been found, but a number of compounds have been used which appear to approach the specifications in varying degree. Among them are thiocyanates, ferrocyanides, sulfates, chlorides, and bromides, and various carbohydrates such as inulin, raffinose, and sucrose. Inorganic ions are unsuitable for extracellular fluid determinations of marine fishes, since they are excreted rapidly by the gills (Smith, 1930). This was also demonstrated with thiocyanate in marine Chondrichthyes (Thorson, 1958), so such substances were not employed on the Osteichthyes.

In my work on Chondrichthyes, the spaces penetrated by inulin, raffinose, and sucrose were compared. These were, in *Squalus acanthias*, respectively, 12.7, 15.2, and 21.2% of the body weight. These volumes are in inverse order to the molecular weights of the three substances: 990, 594, and 342. So that a comparison with at least one bony fish could be made, the same three substances were also compared in the rainbow parrotfish (Table IV), and much the same results were apparent. Sucrose appeared to penetrate more thoroughly in both groups of fish than either inulin or raffinose. Sucrose is considered by many investigators as the substance of choice for higher vertebrates (Robertson, 1953; Wilde, 1945;

Kruhøffer, 1946). It was used in this study, and for purposes of comparison will be employed in future studies on other vertebrate groups.

None of the substances employed by myself and other workers has been shown to penetrate appreciably into the minor fluid compartments. In Chondrichthyes (Thorson, 1958) coelomic, cerebrospinal, and ocular fluids were analyzed for both inulin and sucrose penetration, and the results were negative. In the present work, only the ocular fluid was of sufficient quantity to test, and sucrose was not found in it. This introduces a small error into the extracellular fluid measurements, but the cerebrospinal and coelomic fluids were so small in quantity that they could not be measured directly and it is estimated that the ocular fluid would not make up more than about one-fourth of one per cent of the body weight in any of the species employed. No correction for minor fluids has been introduced in the tables.

It should be borne in mind that the fluid reached by the sucrose includes also lymph, since this is interstitial fluid en route back into the vascular system. It has been impossible to measure lymph separately, so it is included with the interstitial fluid volume.

When the sucrose spaces of the three fresh-water groups are compared, an even more pronounced relationship in the taxonomic series appears than for plasma volume (Table II). The more primitive the group, the higher is its extracellular fluid volume: Teleostei, 14.0%; Holostei, 16.0%; Chondrostei, 18.4%. This continues to be apparent when the comparison is extended to the Chondrichthyes (21.2%) and Agnatha (*Petromyzon marinus*, 23.9%), the most primitive class having the largest sucrose space of all the aquatic classes.

It must be noted that the figure for extracellular fluid volume of the Chondrichthyes is based on only three individual measurements. These are three *Squalus acanthias*, the only chondrichthyans in which sucrose was employed. Otherwise, all extracellular fluid measurements of the four species of Chondrichthyes were made with the use of inulin, which gave lower results (see above). The average inulin space of 31 chondrichthyan fish was 11.9% of the body weight. This figure can be compared directly only with the rainbow parrotfish, in which the inulin space was 11.4% (Table IV). If the sucrose space of the rainbow parrotfish (16.6%, based on eight individuals) is increased by a factor of 11.9/11.4, the result might be used as a calculated sucrose space for Chondrichthyes. This would be 17.3%, as compared with 15.7 for Osteichthyes and 23.9 for *Petromyzon marinus*. The same gradient in extracellular fluid volume is apparent as before, although the difference between Chondrichthyes and Osteichthyes is not as pronounced.

When fresh-water teleosts are compared with marine teleosts, it is seen that the latter have a somewhat larger sucrose space (15.4%) than the former (14.0%). Considering the extreme osmotic dissimilarity of the fresh- and salt-water media, these figures may be more remarkable for their relative closeness than for the slight difference apparent. The difference which exists is probably related, among other things, to the slightly higher osmotic pressure of the blood of marine fish as compared with fresh-water fish (Prosser *et al.*, 1950; Florkin, 1949; Williams, 1951).

There is some variation within all the groups, and, as for plasma and blood

volumes, the rainbow parrotfish has the largest sucrose space of all the marine teleosts, although the difference is not as great in this case. Its sucrose space is also greater than that of any fresh-water teleost and only that of the bowfin and rock sturgeon of the primitive forms exceeds it.

Only one earlier measurement of the extracellular fluid compartment in any aquatic vertebrate is known to the writer. Prosser and Weinstein (1950) determined the thiocyanate space of six yellow bullheads, *Ictalurus natalis*, which are fresh-water teleosts. The average volume was 4.0% of the body weight. In this paper, the average for all fresh-water teleosts was 14.0. The lowest species figure was for the two common suckers (12.2), and the lowest individual measurement was 10.9 for a bigmouth buffalofish. No marine teleost figure was this low, although when inulin was employed (Table IV) a figure of 9.2 was obtained for a single rainbow parrotfish. In view of the great divergence in results, it is inconceivable that the measurements of Prosser and Weinstein could represent chance variation, and it appears probable that the explanation must be sought in differences in substances employed, technique, and interpretation of extrapolation curves.

Interstitial fluid

When the extracellular fluid volume (sucrose space) and plasma volume (T-1824 space) are known, a reasonable approximation of the interstitial (tissue) fluid volume can be reached by subtracting the former from the latter. The lack of information on the minor fluid compartments does not affect this calculation, since the sucrose does not penetrate these compartments. It should be remembered, however, that the figures for interstitial fluid (Tables I and II) include the lymph.

The interstitial fluid volume could not be calculated for every animal employed, since the extracellular fluid and plasma measurements were not always both successful in the same animal. For this reason, the interstitial fluid volume for every species or other taxonomic group was derived from the mean extracellular fluid and plasma figures for that species or group. Therefore, ranges and standard deviations are not given for interstitial fluid in Tables I and II.

The most primitive group (Chondrostei) has the largest interstitial volume (15.9% of the body weight), followed by the Holostei (13.9%); the fresh-water teleosts have the lowest volume (12.2%). The marine teleosts have a somewhat higher volume (13.5%) than the fresh-water teleost species. Comparing the three classes of aquatic vertebrates, Agnatha (*Petromyzon*) has the highest interstitial volume (18.4%), Chondrichthyes has a volume of 15.8%, and the Osteichthyes 13.7%. It is seen, then, that the volume of extracellular fluid relative to the taxonomic groups is reflected in a similar fashion in both of the extracellular sub-compartments, namely plasma and interstitial fluid.

The total circulating fluid, both intra- and extra-vascular, appears to be more plentiful in primitive aquatic vertebrates than in the more advanced, and slightly more plentiful in salt-water forms than in fresh-water.

Total body water

The use of antipyrine for determination of total body water (Soberman, 1950) was unsuccessful in marine Chondrichthyes (Thorson, 1958) and was not at-

tempted in the Osteichthyes. Complete dehydration at 105° C. was chosen as the method most practical for comparison of a wide variety of vertebrates.

In general, the total water content is fairly uniform among osteichthyan species, with two exceptions (Table I). The green moray contained only 63.7% water, appreciably below any other teleost species or the average for teleosts, either fresh-water or marine. This is almost undoubtedly due to the great quantity of oil contained in their bodies. The shortnose gar also had a lower water content (66.7%) than any of the fresh-water species. In this case, the low figure was probably related to the heavy investiture of ganoid scales.

The green moray and gar figures obscure what otherwise would be a comparative picture similar to, although less pronounced than, that for plasma and extracellular fluid. As it is, the fresh- and salt-water teleosts are very close in water content, marine forms having a little less rather than more. Holostei have somewhat less than fresh-water teleosts, even though the bowfin has the highest water content of all species measured. The most primitive group, Chondrostei, however, is true to form in having the highest content of all four groups. The Agnatha (*Petromyzon*) have the highest water content of all three classes (75.6%), as might be expected, and the Chondrichthyes (74.8) are also appreciably higher than the Osteichthyes (71.2).

Intracellular fluid

No method is known to the writer for direct measurement of the intracellular water of a whole organism, and no figures on volumes of the lower vertebrates are known from the literature. In the human the intracellular fluid compartment comprises about 50% of the body weight (Gamble, 1947). When total body water and extracellular water are known, the intracellular fluid volume can be calculated as the difference between the two. Since sucrose does not appear to become distributed in the minor fluids (coelomic, cerebrospinal and ocular), the intracellular fluid, calculated in this manner, includes the minor fluids as well. It has been pointed out that these are almost negligible, and the error thus introduced detracts little from the reliability of the results for comparative purposes.

As for interstitial fluid, the intracellular fluid volume for each group was derived from the mean figures for total water and extracellular water of that group, and ranges and standard deviations are not given in Tables I and II.

Variation between species in calculated intracellular fluid is considerable (Table I), and only when the summaries in Table II are considered is any pattern suggested. In a general way, the relationship of fluid volume to the taxonomic series is here almost exactly reversed from that evident for plasma and extracellular fluid. Of the three fresh-water groups, the two primitive ones have a smaller intracellular volume (Chondrostei, 54.8; Holostei, 54.3) than the teleosts (57.4). The marine teleosts have a smaller intracellular volume (55.4) than the fresh-water teleost species (57.4) and the two primitive classes (Agnatha, 51.7; Chondrichthyes, 53.6) have smaller volumes than the Osteichthyes (55.5).

It was seen in Table I that there was considerable difference between species in total water content, two species, especially, (green moray and shortnose gar) having a considerably lower water content than other species of their groups. This can probably be explained in terms of varying quantities of fats, scales, and

other solids in the bodies of the various species. However, the fact remains that differences in dry weight will affect the fluid measurements when expressed as per cents of total body weight. A more accurate picture of water partitioning can be had if volumes are expressed in terms of total water. Accordingly, in Table V, intra- and extracellular fluid compartments and the latter's sub-compartments, plasma and interstitial fluid, have been converted and expressed as per cents of total body water rather than per cents of body weight as before.

A comparison of these expressions of body fluid volumes points even more strongly than the observations made earlier to the existence of a relationship between body fluid partitioning in aquatic vertebrates and the phylogenetic series, as well as to habitat.

When the figures for extracellular fluid, plasma and interstitial fluid are arranged in order, from smallest to largest, the order of the groups is exactly the same: smallest for fresh-water teleosts, followed by marine teleosts, Holostei and

TABLE V

Body fluid measurements expressed as per cent of total water

	Agnatha (*Petromyzon marinus*)	Chondrichthyes (summary)	Osteichthyes (summary)	Osteichthyes			
				Chondrostei	Holostei	Fresh-water Teleostei	Marine Teleostei
Total water	100	100	100	100	100	100	100
Intracellular fluid	68.4	71.6	78.0	74.9	77.3	80.4	78.3
Extracellular fluid	31.6	28.3	22.0	25.1	22.7	19.6	21.7
Plasma	7.3	7.2	2.8	3.4	2.9	2.5	2.6
Interstitial fluid	24.3	21.1	19.2	21.7	19.8	17.1	19.1

Chondrostei, in that order. Thus, these volumes are highest in the most primitive Chondrostei and lowest in the most advanced teleosts (both fresh-water and marine). The order for intracellular fluid is exactly reversed, the greatest volume being found in the teleosts and the smallest in the primitive Chondrostei. When the comparison of all fluid volumes is extended from the relatively advanced class Osteichthyes to the more primitive Chondrichthyes, in every case the progression is in the same direction as for the groups within the Osteichthyes: larger extracellular, plasma and interstitial volumes; smaller intracellular. The progression is extended again when the most primitive of the three classes, Agnatha, is compared with the others.

It will be noted that the marine teleosts maintain their position in relation to fresh-water teleosts and the other groups in every fluid measurement: the extracellular fluid volume as well as its subdivisions, plasma and interstitial fluid, are slightly higher in marine than in fresh-water teleosts, but lower than those of any other group of aquatic vertebrates. The intracellular fluid is slightly less than that of fresh-water teleosts, but greater than that of any other group considered.

Some of the differences shown in the tables are small and statistically question-

able, and data for Agnatha are somewhat meager, being based on only one species. However, the over-all pattern which emerges appears highly suggestive. When comparisons progress in the direction of primitiveness in the whole series, or from fresh- to salt-water forms within the teleosts, the proportion of total water within the cells for use in the metabolic activities of the protoplasm appears to become less, and a greater proportion of water becomes available outside the cells for transportation of raw materials and metabolites between the cells and the external environment. The more advanced groups, and, concerning habitat, especially the fresh-water teleosts, appear to get along with a smaller amount of circulating fluid, which, then, is presumably used more effectively.

Summary

1. The major body fluid compartments were measured in two species of fresh-water Chondrostei, two species of fresh-water Holostei, three species of fresh-water Teleostei and seven species of marine teleosts. These were compared with previous measurements of an agnathan species and four species of Chondrichthyes.

2. A general correlation was shown between the relative rates of respiratory movements and pulse rates, but neither of these appeared to be related to the taxonomic series. A faster pulse was more characteristic of marine than of fresh-water species.

3. Plasma volume was measured by the dye dilution method, using T-1824. Whole blood volume was calculated from plasma volume and hematocrit. A progressive reduction in plasma and whole blood volume was noted, proceeding from the primitive to the more advanced groups. This is true both among the three classes of aquatic vertebrates and also among the three groups within the Osteichthyes. These volumes were remarkably similar in fresh-water and marine teleost species, although slightly higher in the latter.

4. Extracellular fluid volume was approximated by sucrose dilution. A comparison of inulin, raffinose and sucrose spaces in one species showed most thorough penetration by sucrose and least by inulin, the volumes being in inverse order to the molecular weights of the substances. Sucrose did not penetrate the minor fluid compartments (coelomic, cerebrospinal and ocular fluids) so these are not included in the extracellular data. The minor fluids probably do not exceed one fourth of one per cent of the body weight in any species studied. The sucrose space (extracellular fluid volume), like plasma volume, was found to be greatest in primitive forms and least in the most advanced. The fresh-water and marine forms have similar extracellular volumes, although slightly greater in the latter.

5. Interstitial (tissue) fluid was estimated by subtracting plasma from extracellular fluid. Exactly the same relationship with the taxonomic series and habitat obtained here as for plasma and extracellular fluid.

6. Total body water was measured by complete desiccation at 105° C. It was found to be fairly uniform among osteichthyan species, with two major exceptions, probably related to high oil content of the body and a heavy investiture of scales. The correlation of water content with the taxonomic series is not as clear in this case, although there is a greater water content in the Chondrostei than in the other two groups of Osteichthyes, and in the Agnatha and Chondrichthyes than in

Osteichthyes. Marine species of teleosts have a slightly smaller total water content than fresh-water species.

7. Intracellular water was calculated by subtracting extracellular fluid from total body water. The relationship of this compartment to the taxonomic series is almost exactly reversed from that of plasma and extracellular fluid: a relatively small intracellular volume is characteristic of primitive groups and a larger volume of more advanced groups. Marine teleosts have a somewhat smaller intracellular volume than fresh-water teleosts, likewise a reversal of the relation in plasma and extracellular fluid.

8. The phylogenetic and ecological patterns become somewhat more distinct when the volumes are expressed as per cents of total water rather than of body weight.

9. Some of the differences shown are small and statistically questionable, and comparisons involving Agnatha are based on only one species. However, the over-all pattern suggests that, in general, the more advanced forms, as opposed to the primitive, and the fresh-water teleosts, as opposed to the marine teleosts, function with a smaller proportion of mediating fluid and relatively more protoplasmic water. The circulating fluid thus appears to be utilized more effectively in the advanced aquatic vertebrates and, to a lesser degree, in fresh-water than in marine forms.

LITERATURE CITED

Derrickson, M. B., and W. R. Amberson, 1934. Determination of blood volume in the lower vertebrates by the direct method. *Biol. Bull.*, **67**: 329.

Erlanger, J., 1921. Blood volume and its regulation. *Physiol. Rev.*, **1**: 177–207.

Florkin, M., 1949. Biochemical Evolution. Academic Press, New York.

Gamble, J. L., 1947. Chemical Anatomy, Physiology, and Pathology of Extracellular Fluid. Harvard University Press, Cambridge, Massachusetts.

Gregersen, M. I., and R. A. Rawson, 1959. Blood volume. *Physiol. Rev.*, **39**: 307–342.

Harrison, H. E., 1942. A modification of the diphenylamine method for determination of inulin. *Proc. Soc. Exp. Biol. Med.*, **49**: 111–114.

Keith, N. M., L. G. Rowntree and J. T. Geraghty, 1915. A method for the determination of plasma and blood volume. *Arch. Internal Med.*, **16**: 547–576.

Kruhøffer, P., 1946. Sucrose for extracellular fluid in mammals. *Acta Physiol. Scand.*, **11**: 37–47.

Lennon, R. E., 1954. Feeding mechanism of the sea lamprey and its effect on host fishes. *Fishery Bull., U. S. Dept. Int., Fish and Wildlife Service*, **56** (No. 98): 247–293.

Little, J. M., 1949. A modified diphenylamine procedure for the determination of inulin. *J. Biol. Chem.*, **180**: 747–754.

Martin, A. W., 1950. Some remarks on the blood volume of fish. *In:* Studies Honoring Trevor Kincaid. Univ. of Washington Press, Seattle, Washington.

Prosser, C. L., D. W. Bishop, F. A. Brown, Jr., T. L. Jahn and V. J. Wulff, 1950. Comparative Animal Physiology. W. B. Saunders Company, Philadelphia, Pennsylvania.

Prosser, C. L., and S. J. F. Weinstein, 1950. Comparison of blood volume in animals with open and with closed circulatory systems. *Physiol. Zoöl.*, **23**: 113–124.

Reichert, E. T., and A. P. Brown, 1909. The differentiation and specificity of corresponding proteins and other vital substances in relation to biological classification and organic evolution. *Pub. Carnegie Inst. Washington*, **116**: 1–338.

Robertson, J. D., 1953. Further studies on ionic regulation in marine invertebrates. *J. Exp. Biol.*, **30**: 277–296.

Schiffman, R. H., and P. O. Fromm, 1959. Measurement of some physiological parameters in rainbow trout (*Salmo gairdnerii*). *Canad. J. Zool.*, **37**: 25–32.

SMITH, H. W., 1930. The absorption and excretion of water and salts by marine teleosts. *Amer. J. Physiol.*, **93**: 480–505.

SOBERMAN, R. J., 1950. Use of antipyrine in measurement of total body water in animals. *Proc. Soc. Exp. Biol. Med.*, **74**: 789–792.

SOMOGYI, M., 1930. A method for the preparation of blood filtrates for the determination of sugar. *J. Biol. Chem.*, **86**: 655–663.

THORSON, T. B., 1958. Measurement of the fluid compartments of four species of marine Chondrichthyes. *Physiol. Zoöl.*, **31**: 16–23.

THORSON, T. B., 1959. Partitioning of body water in sea lamprey. *Science*, **130**: 99–100.

TODD, J. C., AND A. H. SANFORD, 1943. Clinical Diagnosis by Laboratory Methods, 10th Ed. W. B. Saunders Company, Philadelphia, Pennsylvania.

WELCKER, H., 1858. Bestimmungen der Menge des Körperblutes und der Blutfärbekraft, sowie Bestimmungen von Zahl, Maass, Oberfläche und Volum des einzelnen Blutkörperchens bei Thieren und bei Menschen. *Zeitschr. rationelle Med.*, **4**: 145–167.

WILDE, W. S., 1945. Sucrose for extracellular fluid in mammals. *Amer. J. Physiol.*, **143**: 666–676.

WILLIAMS, R. T., ED., 1951. The Biochemistry of Fish. Biochemical Society Symposia, No. 6, Cambridge University Press, Cambridge, England.

THE EFFECT OF TEMPERATURE ON THE GROWTH AND SURVIVAL OF SEVERAL MARINE ALGAL SPECIES

RAVENNA UKELES

U. S. Fish and Wildlife Service, Milford, Connecticut

The importance of temperature as an ecological factor limiting the distribution of species is well known. Protozoa and algal species have been found in extreme temperature ranges and have been considered among the most resistant of all organisms. Flagellates have been found living in hot springs at 51° C., algal species at 70° C. to 80° C. and even as high as 90° C. (Davenport, 1897). These examples are interesting, but exceptional, and active stages of "protists" are usually killed as the temperature approaches 45° C. (Hall, 1953). Although information is available on the general characteristics of the biothermal range for many organisms, specific information on the temperature tolerance of individual species frequently is lacking. Such information as is available has often been obtained from nature where the temperature of the natural habitat is subject to fluctuations and, hence, observations may be unreliable (Spector, 1956). Information gained from laboratory studies under defined conditions is an aid in the interpretation of ecological data, just as ecological data may guide laboratory experimentation.

From a practical point of view, interest in the culture of algal and protozoan organisms as food sources for metazoan forms (Gibor, 1956; Davis and Guillard, 1958; Provasoli *et al.*, 1959) makes it desirable to accumulate detailed information on the factors necessary for the survival and growth of particular organisms *in vitro* and *in vivo*. Studies on the temperature characteristics of plankton species are a prerequisite to proposed studies on the temperature tolerance of bivalves (Davis, personal communication). The following is a report of an investigation into the biothermal range of several species of marine plankton organisms that are of importance to filter-feeding animals.

Material and Methods

Temperature requirements of representatives of three classes of phytoplankters were investigated.[1] *Monochrysis lutheri* and *Isochrysis galbana* of the class Chrysophyceae were used, as was *Phaeodactylum tricornutum*, also considered a chrysophyte by Hendey (1954), but which has been used by several workers as *Nitzschia closterium* (Ehrenberg), Wm. Smith forma *minutissima*, (Allen and Nelson, 1910). Lewin (1958) gives the most recent discussion of the taxonomy of *Phaeodactylum tricornutum* and considers it a chrysophyte of the order Bacillariales and proposes a new suborder with *P. tricornutum* as the only known species. Representatives of the class Chlorophyceae included *Dunaliella euchlora*,

[1] The organisms used were in bacteria-free culture, and were from the same cultures used by Davis and Guillard (1958). Source data are, therefore, not repeated here.

Platymonas sp., *Chlamydomonas* sp. of the order Volvocales; *Chlorococcum* sp., *Chlorella* sp. of the order Chlorococcales; and *Protococcus* of the order Chaetophorales. Temperature requirements of the pennate diatoms, *Nitzschia laevis*, *Amphiprora* sp. and *Amphora* sp., and of the centralean diatom, *Actinocyclus* sp., were also investigated.

Glass-covered, 25-milliliter Fernbach flasks were used as culture vessels. These flasks allow for complete illumination and an adequate surface-volume relationship. Each vessel contained 10 ml. of medium and was inoculated with 0.5 ml. of a bacteria-free unialgal stock culture. Stock cultures from which the inocula were made are maintained at 20.5° C. ± 1° C. in a light room. The basal medium consisted of the following: KH_2PO_4, 20 mg.; thiamine HCl, 0.4 mg.; biotin, 2 μg.; vitamin B-12, 2 μg.; $NaNO_3$, 150 mg.; ferric sequestrene (13% Fe), 10 mg.;

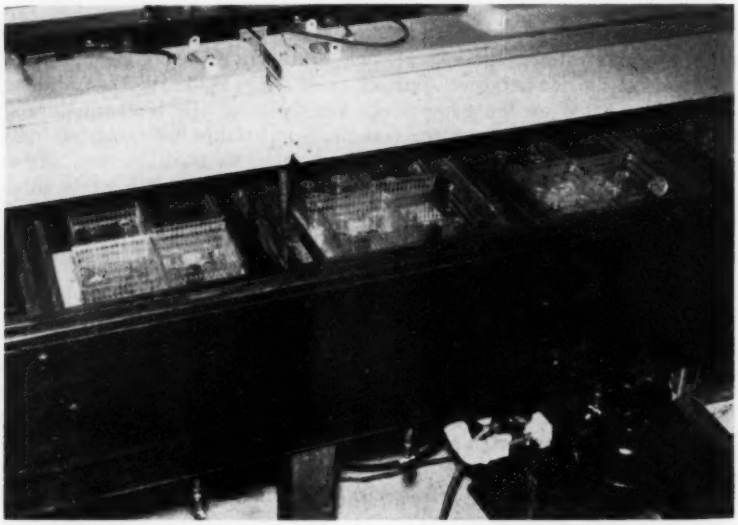


FIGURE 1. Constant temperature apparatus, showing four of the six individual baths, the refrigerating system, the lighting system and the culture vessels.

NH_4Cl, 50 mg.; $CuSO_4 \cdot 5H_2O$, 0.019 mg.; $ZnSO_4 \cdot 7H_2O$, 0.044 mg.; $CoCl_2 \cdot 6H_2O$, 0.022 mg.; $MnCl_2 \cdot 4H_2O$, 0.360 mg.; $Na_2MoO_4 \cdot 2H_2O$, 0.0126 mg.; sea water, 1 liter (22 to 27 ppt). NH_4Cl was eliminated from the basal medium for *I. galbana*.

Experimental temperatures were maintained in a series of constant temperature baths regulated to ± 0.5° C. The culture flasks were submerged on plastic mesh racks into the water baths and incubated at temperatures ranging from 8° C. to 39° C. The apparatus allows for the determination of the effects of six temperatures simultaneously. Light was received from a bank of cool fluorescent bulbs suspended over the water bath to give maximum illumination with a minimum amount of heat (Fig. 1). At periodic intervals cultures were observed for

multiplication of organisms. Where no growth occurred, tests for viable organisms were made by transferring a small inoculum to the basal medium, incubating at an optimum temperature and observing whether multiplication was resumed. In other experiments to determine the effect of temperature on the rate of growth and population density, cultures were incubated in loosely capped calibrated screw-cap test tubes, and growth measured as optical density in a Beckman Model DU Spectrophotometer with test tube adapter assembly.

Results

All of the organisms tested grow normally within the temperature range from 18° C. to 22° C., but there was considerable variation between species, in the minimum and maximum temperatures tolerated (Table I). The maximum tem-

Table I

The growth response of plankton organisms at various temperatures (degrees centigrade)

	No growth	Growth less than control	Growth equal to control at 20.5° C.	Growth less than control	No growth
M. lutheri	8–9°	12°	14–25°	27°	29–35°
I. galbana	8–9°	12°	14–22°	24–25°	27–35°
P. tricornutum	—	—	8–24°	27°	29–35°
D. euchlora	8–9°	—	12–35°	—	39°
Platymonas sp. (#1)	—	8–9°	12–32°	—	35°
Chlamydomonas sp. (Y)	8–9°	—	14–28°	—	32–35°
Chlamydomonas sp. (11/35)	—	8–9°	14–27°	—	—
Chlorella sp. (#580)	8–9°	12°	14–35°	—	—
Chlorella sp. (UHMC)	8–9°	12°	14–29°	—	32–35°
Chlorococcum sp.	—	—	15–30°	—	—
Protococcus sp. (T3)	8–9°	—	12–25°	—	26–32°
Protococcus sp. (T9)	8–9°	—	12–32°	—	35°
Actinocyclus sp.	—	15–17°	18–27°	30°	—
Amphiprora sp.	—	—	8–30°	—	—
N. laevis	—	—	15–24°	27°	30°
Melosira sp.	8–9°	14°	15–24°	—	27–30°
Amphora sp.	—	—	8–30°	—	—

perature for normal growth of the three species of chrysophyte organisms in an eight-day incubation period was 24° C. to 27° C. A temperature of 27° C. resulted in poor growth of *P. tricornutum* and *M. lutheri* and no multiplication of *I. galbana*. At 29° C., *M. lutheri* and *P. tricornutum* also showed no growth. It appeared that *M. lutheri* and *P. tricornutum* cells incubated at 30° C. and *I. galbana* at 27° C. were no longer viable, since growth was not resumed when cultures were transferred to a temperature of 20.5° C. for eight days (Table II). However, with longer incubation periods (25 days) at a favorable temperature, *M. lutheri* cells from a culture that had been incubated at 30° C. did resume normal growth. Similarly, although cells of *I. galbana* incubated at 27.5° C. showed no growth after seven days at an optimum temperature, normal multiplication was resumed by the twenty-fifth day. However, cells incubated at 30° C. did not resume growth.

Microscopic observations indicated that *I. galbana* were most actively moving

TABLE II

Tests for viability at temperatures that inhibited growth. (+ = growth equal to that of controls, ± = growth less than controls, 0 = no growth)

	Initial incubation temperature ° C.	Growth after 7 days	Growth 7 days after transfer to 20.5° C.
Platymonas sp. (#1)	7.5	±	+
	35	0	±
Chlamydomonas sp. (11/35)	7.5	0	+
	35	0	+
D. euchlora	7.5	0	+
	39	0	0
Protococcus sp. (T9)	7.5	0	+
Protococcus sp. (T3)	7.5	0	+
	32	0	0
	39	0	0
Chlorella sp. (580)	7.5	0	+
Chlorella sp. (UHMC)	7.5	0	+
	32	0	0
M. lutheri	7.5	0	+
	30	0	0
	32	0	0
I. galbana	7.5	0	+
	27.5	0	0
	30	0	0
P. tricornutum	28	0	+
	30	0	0
	32	0	0
Melosira sp.	27	0	0
	30	0	0
N. laevis	30	0	±

at 15° C. At 24° C. to 25° C. organisms appeared sluggish and pale, and at 27° C. cells settled out of suspension and only an occasional complete motile cell was observed. *M. lutheri* appeared to be most active at 18° C.; at 27° C., cells seemed sluggish and at 30° C., most of the cells were fragmented and only an occasional complete motile cell was observed.

When subjected to a temperature of 39.5° C., *M. lutheri* and *I. galbana* lost motility within 10 minutes, whereas the only motile chlorophyte tested, *D. euchlora*, remained motile for 45 minutes. In observations made five days after return to a favorable temperature, it appeared as if the viability of *M. lutheri* was affected within 20 minutes and that of *I. galbana* and *P. tricornutum*, within 10 minutes

(Table III). However, observation 13 days after return to normal temperature showed that there was some growth in all *M. lutheri* cultures, even those exposed to 39.5° C. for 65 minutes, in *P. tricornutum* exposed for 35 minutes and in *I. galbana* for 15 minutes.

The Chlorophyceae, as a whole, were more resistant to elevated temperatures than the Chrysophyceae. Of the motile Chlorophyceae, *D. euchlora* was the most temperature-resistant, *Platymonas* sp., the next, and *Chlamydomonas* sp., the least resistant. *D. euchlora* cells, exposed to 39.5° C. for 65 minutes, resumed multiplication within a few days after return to 20.5° C. (Table III), although they showed no growth during the seven-day incubation period at 39° C. (Table II). One strain of *Protococcus* sp. (T9) seemed to be more temperature-resistant than another strain (T3). *Protococcus* sp. (T9) grew at 32° C. but strain (T3) was inhibited at 27° C. (Table I). *Chlorella* sp. (580), *Chlorococcum* sp. and *Protococcus* sp. (T9) were not affected by a 65-minute exposure to 39.5° C.

Growth of the diatoms, *Amphiprora* sp., *Amphora* sp. and *Actinocyclus* sp., was not appreciably affected by temperatures between 8° C. and 30° C., but *N. laevis* and *Melosira* sp. did not grow at 30° C.

Platymonas sp. and *Chlamydomonas* sp. (11/35), incubated for seven days at 35° C., and *N. laevis* incubated at 30° C., were capable of resuming growth when

TABLE III

Thermal inactivation and death times at 39.5° C. as shown by loss of motility and failure to resume growth when returned to 20.5° C. (+ = *growth equal to that of controls*, ± = *growth but less than that of controls*, 0 = *no growth*)

Minutes at 39.5° C.		*M. lutheri*	*I. galbana*	*D. euchlora*	*P. tricornutum*	*Chlorella* sp. (580)	*Chlorococcum* sp.	*Protococcus* sp. (T9)
0**	% motile	100	100	100	*	*	*	*
	growth†	+	+	+	+	+	+	+
5***	% motile	50	25	100	*	*	*	*
	growth†	+	+	+	+	+	+	+
15	% motile	0	0	100	*	*	*	*
	growth†	±	0	+	0	+	+	+
25	% motile	0	0	75	*	*	*	*
	growth†	0	0	+	0	+	+	+
35	% motile	0	0	5	*	*	*	*
	growth†	0	0	+	0	+	+	+
55	% motile	0	0	0	*	*	*	*
	growth†	0	0	±	0	+	+	+
65	% motile	0	0	0	*	*	*	*
	growth†	0	0	±	0	+	+	+

* Species non-motile.
** Controls-returned to 20.5° C. immediately.
*** Time required for cultures to reach 39.5° C.
† Determined 5 days after return to 20.5° C.

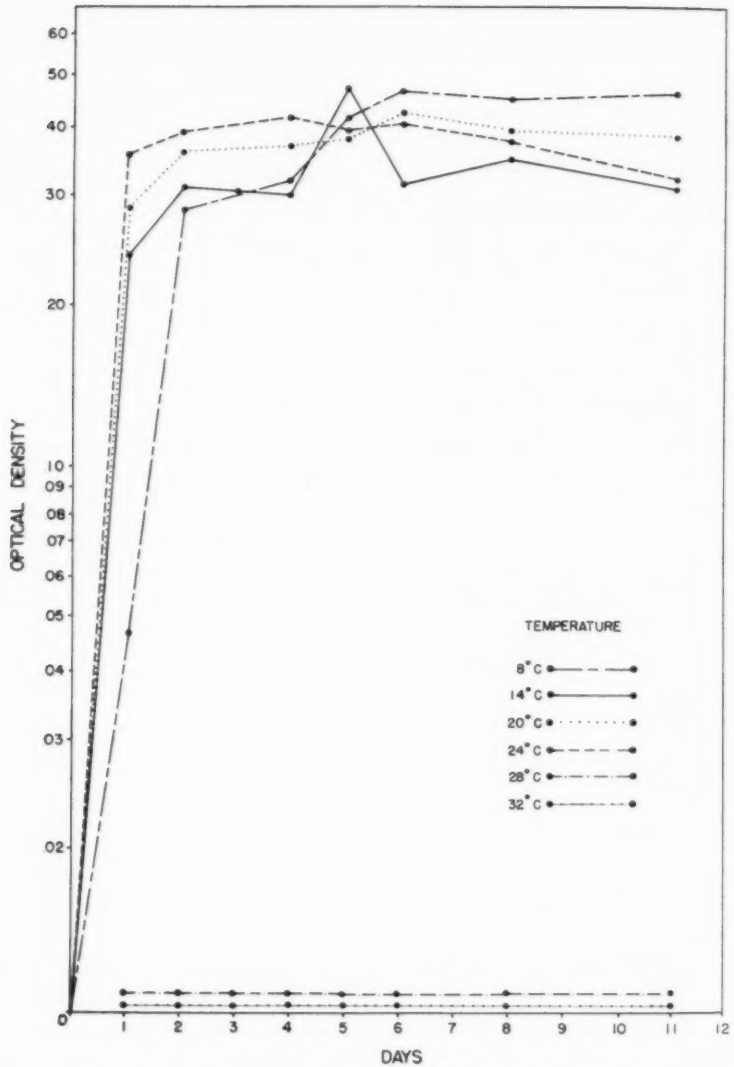


FIGURE 2. The effect of incubation temperatures of 8° C. to 32° C. on the growth of *Phaeodactylum tricornutum.*

transferred to 20.5° C. After a similar incubation period, *D. euchlora* at 39° C., *Protococcus* sp. (T3) at 32° C. to 39° C., *Chlorella* sp. (UHMC) at 32° C. and *Melosira* sp. at 30° C. were apparently not capable of resuming growth when transferred to 20.5° C. (Table II).

In every case in which growth of a species was inhibited by low temperature, growth was resumed when organisms were returned to 20.5° C. (Table II).

Quantitative measurements of the rate of growth and final population levels attained by *P. tricornutum* and *Chlorella* sp. (580) showed that *P. tricornutum* grew significantly faster at 14° C. than at 8° C. but that there was little difference in rates of growth at temperatures from 14° C. to 24° C. The maximum popula-

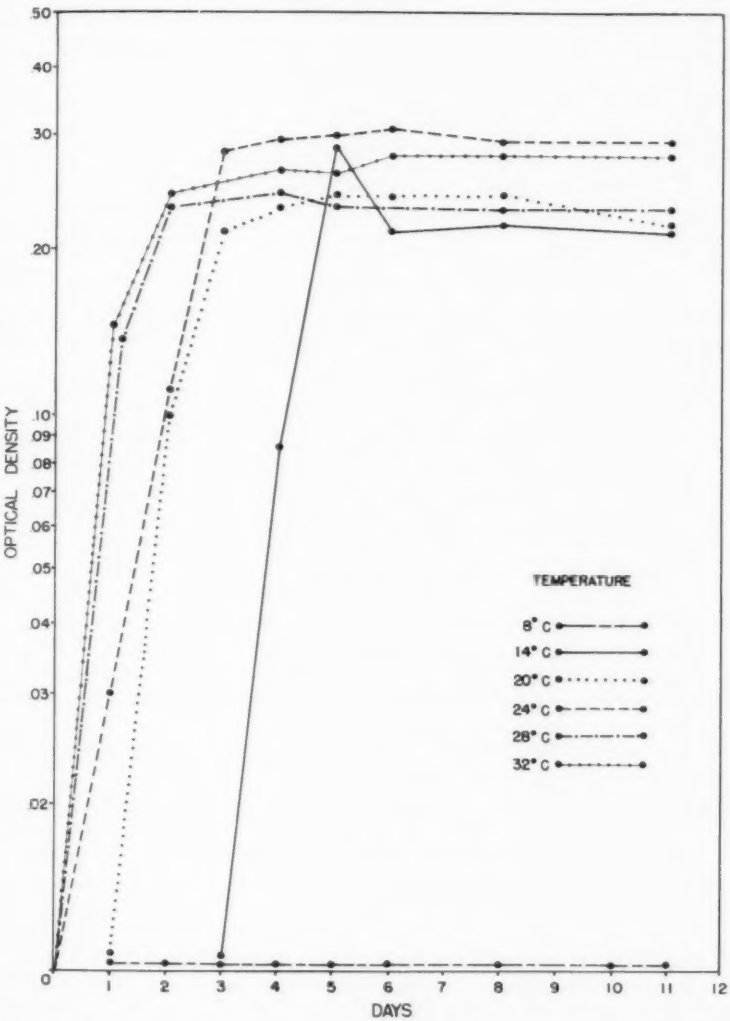


FIGURE 3. The effect of incubation temperatures of 8° C. to 32° C. on the growth of *Chlorella* sp. (580).

tion level attained, before growth leveled off, increased with each increase in temperature from 8° C. to 24° C., but no growth occurred at temperatures of 28° C. or 32° C. (Fig. 2). *Chlorella* sp. (580), conversely, showed no growth at 8° C. but grew rapidly at all temperatures from 14° C. to 32° C. At 14° C. and 20° C. initiation of growth appeared to be delayed and the maximum population level was achieved at 24° C. (Fig. 3).

Discussion

For the chrysophyte organisms tested the upper temperature limits for rapid growth appear to be relatively low, between 24° C. and 27° C. It has been reported that for *Prymnesium parvum*, also a chrysophyte, the upper temperature limit for three strains was 21° C. (Shilo and Aschner, 1953). It may be that this low temperature tolerance is a characteristic of the class. Droop (1953) isolated *M. lutheri* from pools with midday temperatures of 26° C. to 27° C. but, of course, this was not a continuous temperature. Our experiments show that a few individuals of an *M. lutheri* population are capable of surviving at 30° C. and multiply when returned to favorable temperature.

It was found that the maximum temperature for multiplication of *I. galbana* is about 2° lower than that for *M. lutheri* and that the latter species is also more temperature-resistant. In an eight-day incubation period, growth of *I. galbana* was poor at 24° C. and no growth occurred at 27° C. From visual observation, growth appeared to be best at 18° C. to 20° C. Kain and Fogg (1958) found that the optimum temperature for *I. galbana* was between 20° C. and 25° C. and, although they found that there was no growth at 30° C., exposure at this temperature was not lethal. In our experiments we found that cells incubated for eight days at 30° C. did not appear viable even when cells were transferred to an optimum temperature for a period of 25 days. The difference in results may stem from two sources. The English investigators used for their experiments a culture acclimated to an incubation temperature of 25° C., whereas our stock cultures are kept at 20.5° C. ± 1° C. In addition, these investigators used a natural sea water enriched with a soil extract as an incubating medium. The addition of particular crude factors may also alter the maximum tolerated temperature. Recent work has shown that temperature tolerance in the upper temperature regions for flagellates is variable, depending upon the nutritional components of the medium (Hutner *et al.*, 1957). The latter workers showed that, at an elevated temperature, there may be a rise in the requirements for particular vitamins, trace metals or crude factors.

Although the maximum temperature of a marine environment is considered 30° C. to 36° C. (Spector, 1956), the temperature of a particular area may reach higher values, since it is dependent on the absorption of light by an algal mass, or by the depth of a shallow tide pool. Survival of organisms in nature, where high temperatures may be reached, could also be dependent upon the organic content of the water and, thus, the work of Hutner *et al.* (1957) puts a new light on the interpretation of ecological data. Temperature data obtained in different laboratories must also take into consideration the nutritional milieux as well as the past history of the organism. It may be that organisms acclimated to elevated

temperatures will have a higher temperature resistance than other strains maintained at lower temperatures.

Time is also an important factor in determining the biothermal range for a "protist." The end point assay is subject to criticism, since it is dependent upon the survival of an individual organism and an analysis for this variant is being made. The temperature range for growth is narrower than that for survival.

Various theories have been proposed to account for the thermal death of an organism. These include an increase of internal protoplasmic viscosity, vacuolization of the protoplasm due to a release of calcium ion, denaturation of proteins or action on the lipid components of the cell (Heilbrunn, 1952), as well as a nutritional starvation (Hutner *et al.*, 1957). A clue to the nature of thermal death in these organisms may be furnished from studies on their suitability as foods for nutritionally-fastidious metazoan species. Loosanoff (1959) used a mixture of phytoplankton, consisting largely of *Chlorella* sp., to feed *Venus mercenaria* larvae at temperatures of 18° C. to 30° C. with satisfactory results. Experiments show that *Chlorella* sp. is not appreciably affected by a temperature of 30° C. However, in experiments using a mixture of *M. lutheri* and *I. galbana* as food, growth of larvae occurred only up to a temperature of 24° C.; at 27° C., growth was erratic (Davis, personal communication).

This inability of larvae to utilize these foods at temperatures above 27° C. may be due to the fragmentation, clumping and settling out of the food organisms. There is evidence to indicate that physical characteristics of plankton are significant in the utilization as food (Davis and Guillard, 1958). Another interesting possibility is that for the growth of bivalves there may be a nutritional factor or factors provided by the flagellates, that are heat-labile. Because the temperatures are similar, at which growth of larvae and growth of flagellates are inhibited, the possibility suggests itself that it is the heat inactivation of the same nutritional component that is the limiting factor for the growth of the flagellates as well.

LITERATURE CITED

Allen, E. J., and E. W. Nelson, 1910. On the artificial culture of marine plankton organisms. *J. Mar. Biol. Assoc.*, **8**: 421–474.

Davenport, C. B., 1897. Experimental Morphology. Macmillan Co., New York.

Davis, H. C., and R. Guillard, 1958. Relative value of ten genera of micro-organisms as foods for oyster and clam larvae. *Fish. Bull. U. S. Fish and Wildlife Serv.*, **58**: 293–304.

Droop, M. R., 1953. On the ecology of flagellates from some brackish and fresh water rockpools of Finland. *Acta Botanica Fennica*, **51**: 1–52. Helsingfors.

Gibor, A., 1956. Some ecological relationships between phytoplankton and zooplankton. *Biol. Bull.*, **111**: 230–234.

Hall, R. P., 1953. Protozoology. Prentice-Hall Inc., N. Y.

Hendey, N. Ingram, 1954. Note on the Plymouth *Nitzschia* culture. *J. Mar. Biol. Assoc.*, **33**: 335–339.

Heilbrunn, L. V., 1952. An Outline of General Physiology. W. B. Saunders Co., Philadelphia, Pa.

Hutner, S. H., H. Baker, S. Aaronson, H. Nathan, E. Rodrigues, S. Lockwood, M. Sanders and R. Peterson, 1957. Growing *Ochromonas malhamensis* above 35° C. *J. Protozool.*, **4**: 259–269.

Kain, J., and G. Fogg, 1958. Studies on the growth of marine phytoplankton. *J. Mar. Biol. Assoc.*, **37**: 781–788.

LEWIN, J. C., 1958. The taxonomic position of *Phaeodactylum tricornutum*. *J. Gen. Microbiol.*, **18**: 427–432.

LOOSANOFF, V. L., 1959. The size and shape of metamorphosing larvae of *Venus* (*Mercenaria*) *mercenaria* grown at different temperatures. *Biol. Bull.*, **117**: 308–318.

PROVASOLI, L., K. SHIRAISHI AND J. R. LANCE, 1959. Nutritional idiosyncrasies of *Artemia* and *Tigriopus* in monoxenic culture. *Annals N. Y. Acad. Sci.*, **77**: 250–262.

SHILO, M., AND M. ASCHNER, 1953. Factors governing the toxicity of cultures containing the phytoflagellate *Prymnesium parvum* (Carter). *J. Gen. Microbiol.*, **8**: 333–343.

SPECTOR, W. S., 1956. Handbook of Biological Data. W. B. Saunders Co., Philadelphia, Pa.

FOOD OF THE SEA-STAR ASTROPECTEN ARTICULATUS[1]

HARRY W. WELLS, MARY JANE WELLS AND I. E. GRAY

Department of Zoology, Duke University, Durham, North Carolina

It has long been recognized that most sea-stars are carnivorous, devouring whatever living and dead animals they come upon (Hyman, 1955). They have interested biologists because of their depredations on beds of oysters, clams, and mussels in many parts of the world. This concern has centered on the Asteriidae and other asteroids that possess suckers on flexible arms; in these forms, the prey is held or opened by the arms and digestion begins with the stomach everted. The mechanism by which such starfishes successfully attack bivalves has received much attention.

Sea-stars that do not possess suckers on their podia, or long flexible arms, must rely on swallowing their food whole and digesting it internally. This method of feeding is employed by sea-stars of the genus *Astropecten* (Hyman, 1955). Most members of this widespread genus live more or less buried in sandy bottoms, where they ingest large numbers of sand-dwelling animals. Eichelbaum (1910) and Kisch (1958) have reported on the food of *Astropecten irregularis* from northwestern Europe, Hamann (1889) recorded the food of *A. aurantiacus* from the Mediterranean, and Caracelles and Parodiz (1938) reported on molluscs recovered from the stomach of *A. cingulatus* from the Argentine coast. A large portion of the food of these species consists of shelled molluscs. Because organisms are swallowed whole and their shells remain after the soft parts have been digested, the stomach contents of such sea-stars can be analyzed to determine what species have been devoured.

This report discusses the food of *Astropecten articulatus*, a common species off the southeastern coast of the United States.

Materials and Methods

Through the cooperation of local fishermen, 124 sea-stars were obtained from the trawler "MITZI KAY" which operates from Hatteras, North Carolina. They were collected at 4 to 7 fathoms from a sand bottom near Ocracoke Inlet, North Carolina, on January 13, 1959.

The sea-stars were identified as *Astropecten articulatus* (Say) after a careful study of specimens and taxonomic literature dealing with this genus. The purple and orange coloration of living specimens gradually faded on exposure to preservative fluids or light. Because the distribution of spines on the supramarginal plates is of primary importance in the separation of species in this genus, the virtual absence of any supramarginal spines made their identification difficult. However,

[1] Supported by a grant (G-5838) from the National Science Foundation to Duke University, and aided by the Cape Hatteras National Seashore of the National Park Service.

another collection from the same area in late June revealed supramarginal spines on a majority of specimens, in a confusing variability of position. It is well recognized that *A. articulatus* is a variable species, and that supramarginal spines usually develop with increasing age in this group (Verrill, 1915; Döderlein, 1917).

To expose the stomach and its contents, the oral surface was opened by separating the plates along the ambulacral grooves. The contents, often including the thin stomach wall so as to collect any small shells hidden in its folds, were carefully removed. Usually the stomach contents included a sizeable amount of sand, as well as small gastropods, pelecypods, and other animals. These were separated and sorted with the aid of a binocular dissecting microscope.

Extensive use has been made of the mollusc collection of the United States National Museum for comparison and identification of specimens. We wish to thank Drs. H. A. Rehder and J. P. E. Morrison for their generous assistance in the identification of molluscs, and Dr. C. E. Cutress for his assistance in locating *Astropecten* specimens and literature.

Results and Discussion

Of 124 *Astropecten articulatus* stomachs examined, only four were empty. Many contained the remains of a large number of animals. On the average, each stomach contained approximately twelve organisms. The maximum number was 53, two contained 46 each, and nine had 30 or more animals. These numbers exceed the maximum number of organisms recovered from *A. irregularis* by Eichelbaum (1910) or from *A. auranciacus* by Hamann (1889), and compare favorably with those cited by Kisch (1958) for *A. irregularis* from the coast of France. *Astropecten articulatus* is indeed a voracious predator on the inhabitants of the sand bottom community.

Many of the animals recovered were in a surprisingly fresh condition. The soft parts of recently ingested prey were frequently still intact; for example, the eyes of some gastropods could be seen through their transparent shells. Many gastropods retained the operculum, which often aided in identification. Kisch (1958) noted that certain gastropods protected by an operculum may survive more than 48 hours in the stomach of an *Astropecten*. From the stomach of *A. irregularis*, he was able to supply fresh specimens for the description of the soft parts of one gastropod (Fretter, 1956). Similarly, many organisms in fresh condition were recovered from *A. articulatus*. Some of these same species are only infrequently encountered in inshore collections. The ectoproct bryozoan, *Discoporella doma*, recovered from one sea-star still bore the characteristic, long, slender vibracula on its surface. Long-dead colonies of this stony bryozoan have been collected in shallow inshore sand in this region, but they typically lack vibracula and show the effects of considerable abrasion, as described by Maturo (1957).

A total of 91 species was represented in the stomachs examined. This aggregate contains two ectoproct bryozoans, two polychaete annelids, eleven arthropods, three echinoderms, fifty-two gastropods, four scaphopods, and seventeen pelecypods. The most abundant species were the small gastropods, *Acteocina candei*, *Natica pusilla* and *Olivella mutica*. Together these three species account for over 60% of the animals recorded. However, the decapod crustaceans re-

TABLE I

Animals recovered from stomachs of 124 Astropecten articulatus *off Ocracoke Inlet, N. C. January, 1959*

Species	No. of specimens recovered	No. of sea-stars involved
Ectoprocta:		
Discoporella doma (Orbigny)	1	1
Schizoporella unicornis (Johnston)	1	1
Annelida:		
Unidentifiable remains	1	1
Spiochaetopterus oculatus Webster tube	1	1
Arthropoda:		
Cumacea:		
Cyclaspis varians Calman	3	2
Ostracoda:		
Pontocypris edwardsi Cushman	3	2
Pseudocytheretta edwardsi Cushman	14	9
Sarsiella sp.	3	2
Decapoda:		
Sicyonia brevirostris Stimpson	2	2
Crangon septemspinosum Say	3	3
Pagurus annulipes (Stimpson)	1	1
Pagurus corallinus (Benedict)	3	3
Ovalipes ocellatus (Herbst)	13	13
Persephone punctata (Linné)	1	1
Pinnixa sp. juvenile	1	1
Echinodermata:		
Asteroidea:		
Astropecten articulatus (Say)	5	5
Echinoidea:		
Mellita quinquiesperforata (Leske)	15	12
Ophiuroidea:		
Ophiophragmus wurdemanni (Lyman)	1	1
Mollusca:		
Gastropoda:		
Vitrinella beaui Fischer	2	2
Vitrinellidae	1	1
Caecum carolinianum Dall	14	12
Caecum cooperi S. Smith	1	1
Caecum pulchellum Stimpson	19	18
Alaba adamsi Dall	38	20
Finella cerithioides Dall	44	22
Adeorbis supranitidus Wood	12	11
Janthina globosa Swainson	1	1
Epitonium rupicolum Kurtz	2	2
Epitonium multistriatum Say	2	2
Sigatica carolinensis Dall	2	2
Sigatica semisulcata Gray	4	3
Natica pusilla Say	357	89
Anachis iontha Ravenel	5	3
Anachis obesa C. B. Adams	16	14
Anachis ornata Ravenel	8	5
Anachis sayana Rehder	1	1
Mitrella lunata Say	13	11
Nassarius trivittatus (Say)	2*	2
Oliva sayana Ravenel	1*	1
Olivella mutica Say	90	42

* Species represented only by long-dead shells.

TABLE I.—*Continued*

Species	No. of specimens recovered	No. of sea-stars involved
Terebra dislocata Say	1*	1
Niso interrupta Sowerby	2	2
Melanella subcarinata Orbigny	2	2
Rubellatoma rubella Kurtz and Stimpson	5	5
Rubellatoma elata Dall	1	1
Kurtziella limonitella (Dall)	4	4
Brachycythara biconica C. B. Adams	1	1
Mangelia atrostyla Dall	3	3
Mangelia oxia Bush	12	11
Mangelia sp. A	5	5
Mangelia sp. B	6	5
Mangelia sp. C	1	1
Mangelia sp. D	1	1
Cerodrillia sp.	2	2
Turridae	1	1
Acteon punctostriatus C. B. Adams	15	9
Rhizorus oxytatus (Bush)	5	4
Acteocina candei Orbigny	438	57
Cylichnella bidentata (Orbigny)	41	30
Turbonilla (*Turbonilla*) *nivea* (Stimpson)	1	1
T. (*Chemnitzia*) *aequalis* (Say)	17	14
T. (*Pyrgiscus*) *caroliniana* Tuomey and Holmes	1	1
T. (*Pyrgiscus*) *elegantula* Verrill	5	4
T. (*Pyrgiscus*) *areolata* Verrill	10	9
T. (*Pyrgiscus*) *interrupta* (Totten)	35	24
T. (*Pyrgiscus*) *mighelsi* Bartsch	1	1
Odostomia (*O.*) *modesta* (Stimpson)	3	2
O. (*Chrysallida*) *seminuda* C. B. Adams	4	2
O. (*Menestho*) *impressa* Say	2*	1
Cavolina longirostris Lesueur	2	2
Scaphopoda:		
Cadulus carolinensis Bush	9	7
Dentalium occidentale Stimpson	13	9
Dentalium eboreum Conrad	3*	3
Dentalium sowerbyi Guilding	5	5
Pelecypoda:		
Nucula proxima Say	1	1
Anadara ovalis (Bruguière)	1	1
Aequipecten gibbus (Linné)	2	2
Anomia simplex Orbigny	3*	3
Phacoides (*Parvilucina*) n. sp.	11	10
Bornia longipes Stimpson	21	15
Mercenaria campechiensis (Gmelin)	1	1
Chione intapurpurea Conrad	5	5
Gouldia cerina C. B. Adams	2	1
Macrocallista maculata (Linné)	4	2
Dosinia discus Reeve	3	3
Tellina americana Dall	2	2
Tellina sp.	1	1
Strigilla mirabilis Philippi	1	1
Ervilia concentrica Gould	7	5
Corbula swiftiana C. B. Adams	3	3
Pandora arenosa Conrad	14	6

covered probably represent a volume of digestible tissue equal to or greater than that contained in the above-mentioned gastropods. Legs, chelae, and entire bodies of the portunid, *Ovalipes ocellatus*, constituted the greatest portion of this decapod fraction. All species identified from *Astropecten articulatus* stomachs are listed in Table I, with the number of specimens of each and the number of sea-stars involved.

Although the echinoids and certain of the pelecypods were represented by juvenile specimens, the great majority of organisms collected from stomachs were adults, near the maximum size attained by each species. Exceptions to this generality include young sand-dollars, *Mellita quinquiesperforata* (less than 10 mm. in diameter), and juvenile clams, *Chione intapurpurea* and *Macrocallista maculata* (less than 6 mm. in length). Most of the other molluscs recovered were of a similar, minute size, being less than 6 mm. in length. Of the three most abundant species in this collection, *Natica pusilla* and *Acteocina candei* averaged about 3 mm., and *Olivella mutica* averaged about 4 mm. in length.

Several molluscs in this collection have not been taken in the inshore waters of the region. Notable examples are *Natica pusilla, Sigatica semisulcata, Adeorbis supranitidus, Alaba adamsi, Finella cerithioides* and *Acteocina candei* among the gastropods; the new species of *Phacoides, Macrocallista maculata, Pandora arenosa* and *Corbula swiftiana* among the pelecypods; and *Cadulus carolinensis* among the scaphopods.

In a few cases, indicated by an asterisk in Table I, shells were recovered which showed signs (discoloration, worn broken edges) that they were not fresh. It seems likely that these shells came from fossil or sub-fossil deposits in the area, particularly for those species which are not represented alive in the area today. This applies, for example, to *Nassarius trivittatus*, the New England Nassa, which has not been collected alive south of Chesapeake Bay. (Pearse, Humm and Wharton (1942) confused this species at Beaufort, N. C., with a form of *N. vibex*.) Evidently, *Astropecten articulatus* ingests mollusc shells whether they are occupied by the original inhabitant or not. Of course, some of these dead shells may have contained hemit crabs (*Pagurus* species) and thus have contributed to the sea-star's nutrition.

Several other species which appear to be out of place among the bottom fauna are represented. The purple sea-snail, *Janthina globosa*, and the pteropod, *Cavolina longirostris*, are typically pelagic, the former being carried about near the surface by its prey or by a foam raft of its own making, and the latter by active swimming. With death, these species fall to the ocean floor where they can be eaten by *Astropecten articulatus* or any other scavenger. In addition, a number of fish scales were contained in the stomachs. While some could have been ingested while the starfish were in the fish trawl, most fish scales had undoubtedly been picked up from the sand bottom.

Astropecten articulatus could be called cannibalistic in view of the presence in their stomachs of small specimens and fragments of the same species. Most of these had been ingested as fragments of broken sea-stars, perhaps left by the passage of other trawlers, although a few small specimens had been swallowed entire by larger starfish. Kisch (1958) noted such cannibalism in *Astropecten irregularis* off the coast of France, and the ingestion of other echinoderms is not an unusual

practice in the genus *Astropecten*. According to Eichelbaum (1910), other echinoderms compose a major part of the diet of sea-stars of the genus *Luidia*.

Because the gastropod genera *Niso* and *Melanella* are recognized as ectoparasites of echinoderms, the occurrence of specimens of each raised the question of whether these species might be parasitizing *A. articulatus*. However, it should be noted that the asteroid *Luidia clathrata*, the echinoids *Mellita quinquiesperforata*, *Encope emarginata*, and *Moira atropos*, and the holothurian *Thyone briareus* are all present in this immediate area. Unless one can observe the feeding activities of these gastropods, no decision can be made as to which echinoderm species they parasitize.

Kisch (1958) listed sixteen molluscs recovered from *Astropecten irregularis* that represented new records for the area collected (Roscoff, France). The most notable novelties among the molluscs of the present collection are a new pelecypod (to be described elsewhere), and *Sigatica semisulcata*, a rare gastropod previously reported only from the west coast of Florida. Because the taxonomy of turrid gastropods is in a state of confusion, additional undescribed species may be represented in the Turridae of this collection.

Many of the molluscs represented have been treated in the account of species dredged by the United States Fish Commission steamer "ALBATROSS" in the Cape Hatteras region in the 1880's (Bush, 1885). In the publication, several species were described as new from this area. Their appearance in this collection serves as an indication of the value of including an analysis of starfish stomach contents among sampling methods for benthic organisms. From all indications, *Astropecten articulatus* appears to be a non-selective feeder, its stomach contents reflecting the faunal composition of the offshore sand bottoms frequented by it. Indeed, this species can serve the marine biologist as a convenient tool for the study of bottom fauna, as a dredge which samples indiscriminately the abundant minute animals that live in sand.

This generalization evidently applies equally well to other species of *Astropecten* that inhabit sand. If one compares the lists of food organisms recovered from the stomachs of various species of *Astropecten* in different parts of the world, a striking similarity becomes evident. There is a close correspondence in the types of molluscs represented, exemplified by the presence of representatives of the same genera, from widely separated localities. Making allowances for different interpretations of the latitude of certain genera, one finds that the principal difference in these lists of molluscs is at the species level. These specific differences are essentially geographic variations on a basic form, that reflect the geographic affinity of a particular sand-bottom fauna. Therefore, the food organisms recovered from stomachs of different species of *Astropecten* probably are comparable samples of the sand-dwelling fauna of their respective regions. The genus *Astropecten* may serve as a tool for comparative studies of sand-bottom communities in different parts of the world.

Summary

The stomach contents of 124 *Astropecten articulatus* have been identified and discussed. This starfish ingests great numbers of small sand-dwelling organisms. Ninety-one species of invertebrates were recovered, of which 73 were molluscs. The gastropods *Acteocina candei*, *Natica pusilla* and *Olivella mutica* comprised

over 60% of the animals recovered. *Astropecten articulatus* apparently ingests whatever animals or shells it encounters. Because it effectively samples the fauna of offshore sand bottoms, its use is recommended as a collection tool for the study of this community.

LITERATURE CITED

BUSH, KATHERINE J., 1885. Additions to the shallow-water Mollusca of Cape Hatteras, N. C., dredged by the U. S. Fish Commission Steamer "Albatross" in 1883 and 1884. *Trans. Conn. Acad. Arts Sci.*, **6**: 453–480.

CARACELLES, A., AND J. J. PARODIZ, 1938. Moluscos del contenido estomacal de *"Astropecten cingulatus"* Sladen. *Physis.*, **12**: 251–266.

DÖDERLEIN, L., 1917. Die Asteriden der Siboga-Expedition. I. Die Gattung *Astropecten*. *Siboga Exped. Monogr.* **46a** (Livr. 81): 1–191.

EICHELBAUM, E., 1910. Über Nährung und Ernährungsorgane von Echinodermen. *Wissensch. Meeresuntersuch., Abt. Kiel*, **11**: 187–275.

FRETTER, VERA, 1956. The anatomy of the prosobranch *Circulus striatus* (Philippi) and a review of its systematic position. *Proc. Zool. Soc. Lond.*, **126**: 369–381.

HAMANN, O., 1889. Beiträge zur Histologie der Echinodermen. *Jena. Zeitschr. f. Naturwiss.*, **23**: 233–384.

HYMAN, LIBBIE H., 1955. The Invertebrates: Echinodermata (Vol. 4). McGraw-Hill Book Co., Inc. New York. 763 pp.

KISCH, B. S., 1958. *Astropecten irregularis* précieux auxiliare du malacologiste. *Bull. Cent. Étud. Rech. Sci. Biarritz*, **2** (1): 9–15.

MATURO, F. J. S., 1957. A study of the bryozoa of Beaufort, North Carolina, and vicinity. *J. Elisha Mitchell Sci. Soc.*, **73**: 11–68.

PEARSE, A. S., H. J. HUMM AND G. W. WHARTON, 1942. Ecology of sand beaches at Beaufort, North Carolina. *Ecol. Monogr.*, **12**: 135–190.

VERRILL, A. E., 1915. Report on the starfishes of the West Indies, Florida and Brazil. *Univ. Iowa Bull. Lab. Nat. Hist.*, **7** (1): 3–232.

STUDIES ON THE SEXUAL ORGANIZATION OF THE RHIZOCEPHALA. III. THE MODE OF SEX-DETERMINATION IN PELTOGASTERELLA[1]

RYUZO YANAGIMACHI[2]

Zoological Institute, Faculty of Science, Hokkaido University, Sapporo

All the known Rhizocephala except a few species of the family Sylonidae have been hitherto described as hermaphroditic animals. In the first of this series of papers (Ichikawa and Yanagimachi, 1958) it was reported that *Peltogasterella socialis* Krüger, which had been regarded as one of the representative species of the Rhizocephala, is not a hermaphrodite, but a female fertilized by larval cypris males.

A similar sexual organization was demonstrated in *Peltogaster paguri* Rathke and in *Sacculina senta* Boschma (Ichikawa and Yanagimachi, 1960). The sexes of these rhizocephalans are completely separate. The mode of sexual determination in the early stages of *Peltogasterella socialis* Krüger is discussed in this paper.

According to Delage (1884) and Smith (1906), the cypris larvae of *Sacculina* which affix themselves to the host-crabs do not differ morphologically from the larval males that attach around the mantle opening of juvenile parasites. Reinhard (1942) stated that all the cypris larvae of *Peltogaster paguri* are sexually undetermined and undifferentiated. The larvae which attach directly to the host-crabs develop into hermaphrodites. The larvae which fix to the juvenile hermaphrodites become males. Veillet (1943, 1945) reported two kinds of cypris larvae in *Triangulus galatheae* (Syn. *Lernaeodiscus galatheae*) which were considerably different in size. He surmised that the large cypris larvae become hermaphrodites and the smaller cypris larvae function as larval males. According to him, the larger cypris larvae developed from large eggs and the smaller cypris larvae developed from small eggs. Thus, Veillet suggested that the sexes of *Triangulus galatheae* are determined in the egg stage.

In this investigation, it will be reported that the sex of *Peltogasterella*, as the sex of *Triangulus*, is determined at the beginning of development.

Material and Methods

The material used in this investigation was *Peltogasterella gracilis* (Boschma) (syn. *Peltogasterella socialis* Krüger; *cf.* Reischman, 1959) collected from the shore of Oshoro Bay on the west coast of Hokkaido. In this locality the host of this rhizocephalan is almost exclusively the hairy sea-shore hermit crab, *Pagurus lanuginosus* de Haan.

Rearing of the adult and the larvae. It is generally believed that the rearing of the rhizocephalans under laboratory conditions is difficult. However, the author

[1] Contribution No. 473 from the Zoological Institute, Faculty of Science, Hokkaido University, Sapporo, Japan.

[2] Present address: The Worcester Foundation for Experimental Biology, Shrewsbury, Massachusetts.

could rear both adult and larval forms of *Peltogasterella* without difficulty. The successful rearing of the adult form was effected by keeping the host-crabs in a healthy environment. The vessels in which the parasitized crabs were kept had a diameter of 15 cm. and a depth of 10 cm. One parasitized crab, after being detached from shell, was placed in each vessel with the level of sea water sufficient to cover the crab. Each vessel was covered with a glass plate to prevent evaporation of the water. Room temperature was not regulated. The crabs and their parasites were not very sensitive to temperature changes. The crabs survived for a long period of time without food. However, it is better to feed them pieces of mussel or clam at intervals. Food debris and the excrements of the crabs were carefully discarded. The sea water in the vessel was renewed at frequent intervals. The females of *Peltogasterella* on the crabs, under favorable conditions, remained alive for about two months. During this period they emitted several batches of nauplius larvae at regular intervals of two weeks (at 20° C.).

The nauplius larvae emitted from the females were successfully reared in clean sea water when the surface of the water was covered with a film of cetyl alcohol. If the film of alcohol was not employed, a number of larvae adhered to the surface of the water and eventually perished. For the details of the method by which the author succeeded in rearing the larvae, readers are referred to Yanagimachi (1961).

Demonstration of chromosomes of mature eggs. The simple aceto-orcein squash technique was adopted to demonstrate the chromosomes. The procedures were as follows. (1) Detach mature females from the hosts and break the mantle and the wall of ovaries by the use of scissors or needles. (2) Collect the eggs with a pipette and stain them with aceto-orcein for 15 to 30 minutes. The aceto-orcein was prepared according to LaCour (1941). (3) Place the stained eggs on slide with a few drops of aceto-orcein, put a coverslip over the specimens, and blot excess stain away. Squash the stained larvae by exerting careful and controlled pressure on the coverslip. (4) Seal the coverslip with balsam-paraffin (1:1). The preparations thus made remained in perfect condition for months.

Results

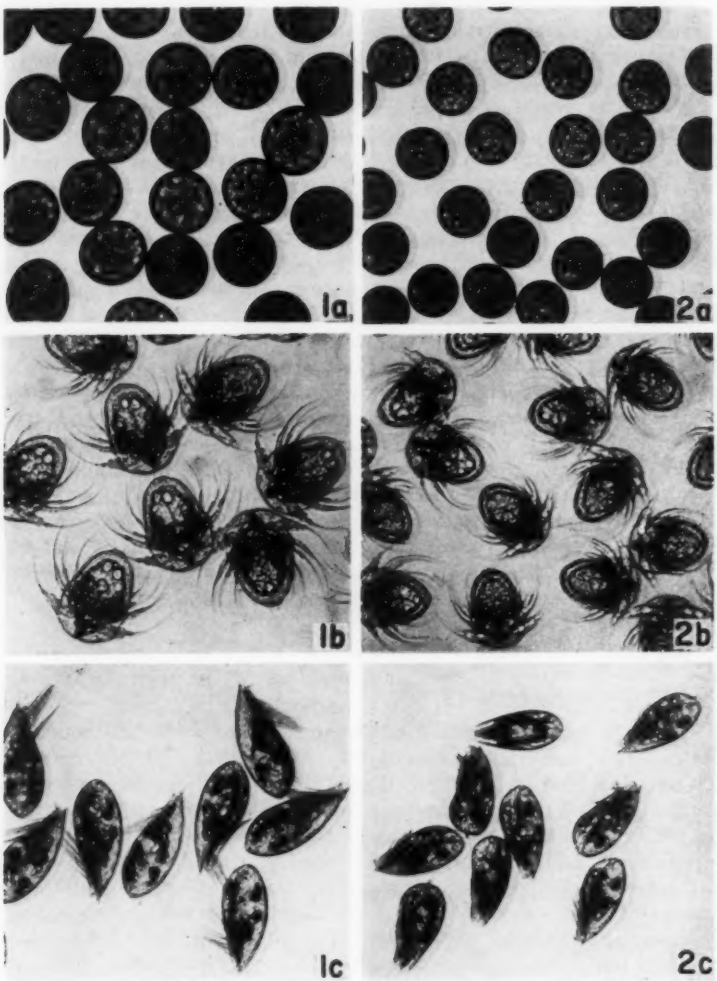
1. *The dimorphism of eggs and larvae*

(*a*) When a number of adult females of *Peltogasterella* had been reared, all females on some host-crabs produced only large eggs. On the other hand, all females on other host-crabs produced only small eggs. In the maternal mantle cavity (brood pouch), these two types of eggs developed at the same rate and reached the nauplius stage after two weeks (at 20° C.). The large eggs developed into large nauplii, and the small eggs developed into small nauplii. When emitted from the maternal mantle cavity, the nauplii swam actively in sea water and underwent successive moults in 4 to 5 days. The large nauplii metamorphosed into large cypris larvae, and the small nauplii developed into small cypris larvae.

Figures 1 and 2 are photomicrographs of the eggs and larvae of the large and small types derived from the females on two different host-crabs. The measurements of these eggs and larvae are summarized in Figure 3.

The two types of eggs, *viz.*, the large and small eggs, so far as could be ascertained, did not show any distinct structural difference. They differed only in size.

In the nauplius stage, the two types of larvae were identical in structure. They were distinguishable only by their size. In the cypris stage, they were different not only in size but also in structure. The most pronounced structural difference was found in the antennules which function as organs of attachment. Figures 4



FIGURES 1a–c. Eggs and larvae of large type, derived from female *A*. (a) Mature unfertilized eggs. (b) Newly hatched nauplii. (c) Cypris. Magnification: × 55.

FIGURES 2a–c. Eggs and larvae of small type, derived from female *B*. (a) Mature unfertilized eggs. (b) Newly hatched nauplii. (c) Cypris. Magnification: × 55.

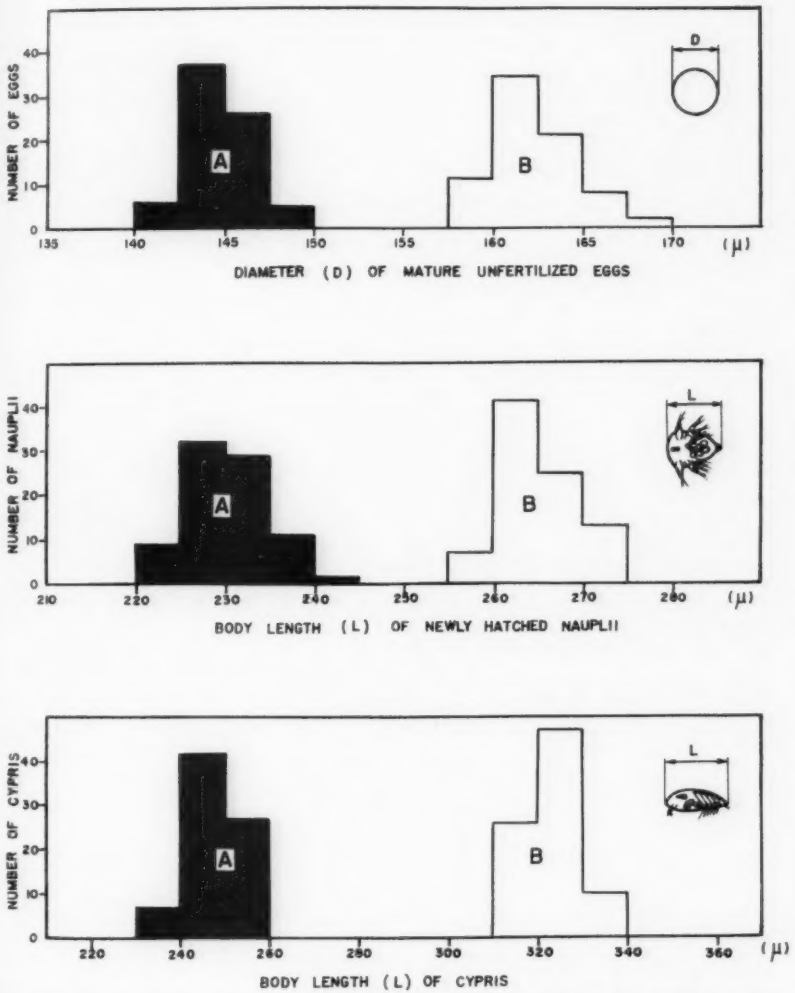


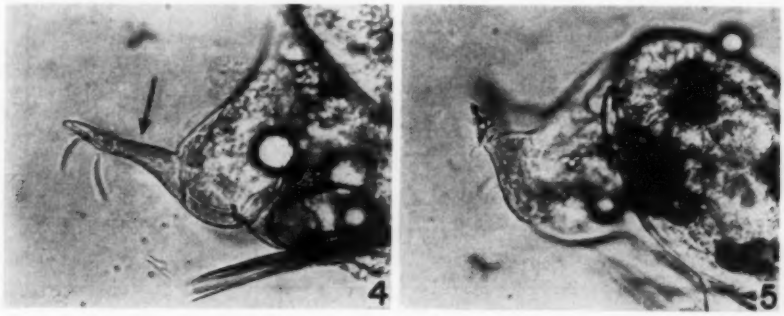
FIGURE 3. Range in size of eggs and larvae derived from two different females, *A* and *B*.

and 5 show the antennules of large and small cypris larvae. The penultimate segment of the antennule of the large cypris is comparatively long and slender (Fig. 4), whilst the corresponding part of the small cypris is greatly diminished or absent (Fig. 5).

(*b*) During its lifetime, an adult female emits several batches of larvae. Experiments were undertaken to know if one female always produces either large or small larvae.

A crab carrying adult females of *Peltogasterella* on its abdomen was placed in a vessel of sea water. All the females except one were mechanically detached. Then, the successive batches of nauplius larvae expelled from this remaining female were examined. Five experiments of this type were conducted. It was found that one female always produces either large or small larvae.

(*c*) *Peltogasterella gracilis,* in common with other species of its genus, occurs gregariously on one host. Do females of *Peltogasterella* on a single host emit both large and small larvae? Table I summarizes the measurements of the larvae and of the eggs derived from the females on six different host-crabs. It may be seen from this table that the females on one host produce larvae of one type—either large or small. The author examined the females on 25 different host-crabs. Females on 11 crabs (2♀ and 9♂) produced exclusively large larvae and those on 12 other crabs (7♀ and 5♂) produced small larvae. The females on the remaining two crabs, on the other hand, produced both large and small larvae. The data on these two exceptional females will be presented later.



FIGURES 4 and 5. Comparison of the antennules of the large and small cypris larvae. The cypris larvae were slightly compressed beneath coverslip. (4) Large cypris; an arrow indicates the penultimate segment. (5) Small cypris. Magnification: × 300.

2. *Identification of the sex of larvae*

As has been noted, Veillet (1943, 1945) found large and small larvae in *Triangulus galatheae*. He surmised that the large cypris larvae affix to the body of the host-crabs and develop into hermaphrodites, and that the small ones function as larval males. The present author came to a different conclusion about *Peltogasterella gracilis*. The experiments on which this conclusion is based are here cited.

(*Experiment 1*). On Septemeber 28, 1959, large nauplii were emitted from three females on a host-crab. These larvae were placed in a vessel of sea water and reared. They metamorphosed into large cypris larvae on October 3. On the evening of October 6, two young crabs (*Pagurus lanuginosus* of 3 mm. carapace length) were put into the vessel in which the cypris larvae were actively swimming. At the same time, a large crab (*Pagurus lanuginosus* of 10 mm. carapace length)

TABLE I

Measurements of eggs and larvae derived from Peltogasterella females on six different host-crabs

Host (*Pagurus lanuginosus*)		Parasite (*Peltogasterella gracilis*)				
Sex Carapace length	Individual	Size (length of external sac mm.)	Mean diameter of eggs (μ)*	Range of body length of nauplii (μ)**	Range of body length of cypris larvae (μ)#	Type of cypris##
Male 6 mm.	a	7	167	265–283	312–329	large
	b	7	163	250–270	312–328	large
	c	7.5	—	—	295–325	large
	d	7	—	—	312–328	large
	e	7	—	257–270	—	—
Male 11 mm.	a	7.5	161	256–276	312–336	large
	b	7	—	—	295–315	large
	c	7	—	—	312–325	large
	d	6.5	—	—	312–320	large
	e	7	—	—	311–332	large
Male 15 mm.	a	5	170	260–274	309–330	large
	b	5	168	263–268	312–329	large
	c	6	173	—	311–329	large
	d	6	—	—	312–329	large
	e	4	—	260–290	295–320	large
	f	5	—	260–276	—	—
Female 12 mm.	a	6.5	143	225–242	242–260	small
	b	7	140	219–240	225–245	small
	c	7	—	225–242	230–252	small
	d	6	—	225–242	—	—
Male 13 mm.	a	9	144	222–243	242–260	small
	b	8	140	212–234	242–250	small
	c	8.5	—	210–235	250–275	small
	d	8	—	—	242–260	small
	e	9	—	—	234–260	small
	f	6	—	—	235–255	small
Female 13 mm.	a	7.5	142	216–242	242–260	small
	b	7	145	220–240	234–256	small
	c	7	—	—	234–260	small
	d	6.5	—	207–225	—	—
	e	7	—	219–230	—	—

* Mature unfertilized eggs which had just been released from the ovary into mantle cavity (brood pouch) were measured. The measurement was made on about 50 eggs.

** The measurement was made on 30–50 newly hatched nauplii which had not yet undergone their first moulting. The body length means the distance between the frontal margin of the carapace and the tip of the caudal spine.

\# The measurement was made on 30–50 recently metamorphosed cypris larvae. The body length means the distance between the anterior and the posterior ends of the carapace.

\#\# Types of cypris larvae were identified by measuring the body size and examining the structure of antennules.

carrying juvenile females of *Peltogasterella* on its abdomen was placed in the same vessel. The number of juvenile females on the crab was 15, and they varied from 2.5 to 3 mm. in length of the external sac-like portion. In every respect they were identical to those females which had recently emerged from the crab's abdomen. On the next morning, the three crabs were removed from the vessel and examined under a binocular dissecting microscope. Five cypris larvae were found attached to the mantle opening of four juvenile females of *Peltogasterella*. Two cypris larvae were about to inject their internal cellular contents into the mantle cavity of the juveniles (Fig. 6). Three cypris larvae, on the other hand, had already injected their cellular contents into the juveniles. No cypris was detected on the surface of the crab's body, although careful and repeated examinations were carried out.

(*Experiment 2*). On October 1, small nauplii were discharged from five females on a host-crab. They were placed in a vessel of sea water and reared. On October 5, they metamorphosed into small cypris larvae. On the evening of

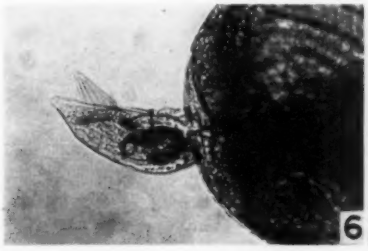


FIGURE 6. A large cypris fixed at the mantle opening of a juvenile female. Magnification: × 80.

FIGURE 7. A small cypris fixed at the base of a seta on the carapace of a host-crab. Magnification: × 80.

October 7, two young crabs (*Pagurus lanuginosus* of 3 to 4 mm. carapace length) were put into the vessel in which the small cypris larvae were swimming. At the same time, a large crab (*Pagurus lanuginosus* of 13 mm. carapace length), infested by 13 juvenile females of *Peltogasterella*, was put into the vessel. The next morning, the three crabs were removed from the vessel. On careful examination, several cypris larvae were found attached to the body of the young crabs. These cypris larvae were invariably affixed to the base of the setae on the crab's body. The setae on the carapace were the most frequent site of fixation (Fig. 7). No cypris was found at the mantle opening of the juvenile females of *Peltogasterella*.

Fifteen experiments of these types have been conducted, with the result that the large cypris larvae always attach to the mantle opening of the juvenile females of *Peltogasterella* and never fix on the body of the host-crab. The small cypris larvae search for the host-crabs and affix at the base of setae, without being attracted to the juvenile females. From these facts it is concluded that the cypris larvae of the small type are prospective females and those of the large type function as larval males.

3. *Morphological distinction in the male-producing and female-producing females*

As stated in the first section, the females of *Peltogasterella* on one host usually produce larvae of the same type, *i.e.*, either large or small larvae. From the results of the experiments described, it is now certain that the larvae of small type are prospective females while those of large type function as males. Then, it may be said that the females on a host are regularly "female-producing" or "male-producing."

Now, a question arises as to whether the female-producing and the male-producing females are morphologically different. A number of living specimens of both female-producing and male-producing adult females were examined to obtain the answer to this question. The external appearance of the sac-like portion, the position of the stalk, the site and the shape of the colleteric glands, and the structure of the mantle opening were compared, but no distinct difference was detected. Some specimens of the female-producing and the male-producing females were cut in serial sections and examined. No discernible difference in structure of various organs of the two types of the adult females was detected. The observations of the author reveal no evident morphological difference between the male-producing and the female-producing females.

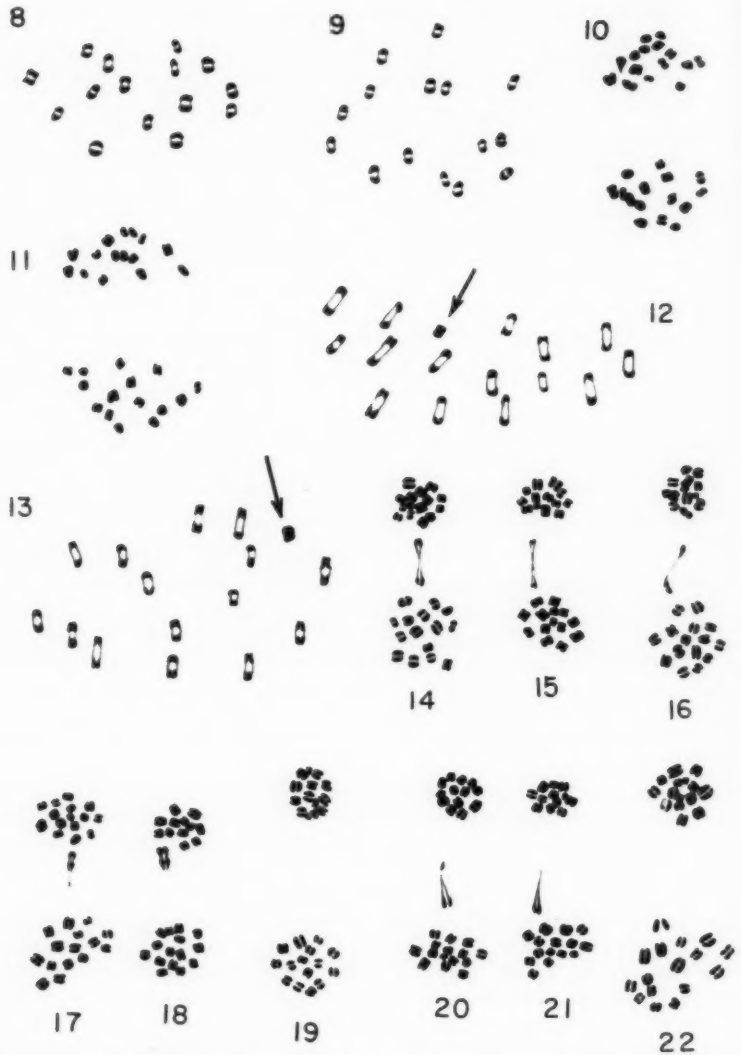
4. *The differences in the chromosomes of the male-producing and the female-producing eggs*

The eggs of *Peltogasterella* begin maturation division while they are still in the ovary. Having reached the stage of the first maturation division metaphase, the eggs are expelled through the colleteric glands into the mantle cavity of the female. The first maturation spindle then passes into telophase and the first polar body is extruded, the second polar body being immediately formed without reconstruction of the nucleus. The penetration of the sperm into the egg occurs immediately before the second polar body is extruded (Shirase and Yanagimachi, 1957).

When the author examined the metaphase plate of the first maturation division of the large male-producing eggs, 15 bivalent chromosomes were observed (Figs. 8 and 9). In the anaphase of this division, these chromosomes segregated equally and passed into the two poles (Figs. 10 and 11). In the metaphase plate of the second maturation division, 15 univalent chromosomes were counted.[3]

The chromosomal arrangements were different in the small female-producing eggs. In the metaphase plate of the first maturation division, a univalent chromosome was always found with 15 bivalent chromosomes (Figs. 12 and 13). This chromosome showed a peculiar behavior at the later stage of the first maturation division. It remained in the middle of the spindle after the other chromosomes had passed to the pole (Figs. 14–16). It was greatly stretched in the axis of the spindle and had the appearance of a body under considerable tension. In most cases it showed a conspicuous longitudinal split which was so distinct that the halves resembled two parallel lagging chromosomes. Only in the late anaphase of the division did this peculiar univalent chromosome pass to one pole of the spindle (Figs. 17–22). In the metaphase plates of the second maturation division

[3] Thirty to forty chromosome counts have been made for each of the represented stages.



FIGURES 8–22. Chromosomes of the large and the small eggs. Magnification: × 1500. Camera-lucida drawings.

FIGURES 8–9. Chromosomes of the first maturation division metaphase of the large male-producing egg.

FIGURES 10–11. Chromosomes of the first maturation division anaphase of the large male-producing egg.

FIGURES 12–13. Chromosomes of the first maturation division metaphase of the small female-producing egg. A univalent chromosome (indicated by an arrow) is found beside 15 bivalent chromosomes.

15 and 16 univalent chromosomes were counted. No lagging chromosomes were detected in the course of second maturation division.

5. *On a few exceptional females*

Of 25 parasitized crabs that the author reared, two were found to bear unusual females which produced both large and small larvae. The data concerning these females are here presented.

(*a*) *Five females on a host-crab* (♀) *of 12 mm. carapace length.* All of these females measured from 8.5 to 9.5 mm. in length of the external sac-like portion. One of these emitted a batch of nauplius larvae on October 10. When these nauplii were measured immediately after hatching, 90% of them had a body length of 225 to 240 μ. The remaining 10% of the larvae, on the other hand, measured from 250 to 260 μ in length. Thus, the nauplii emitted from this female consisted of many small larvae and a small number of large larvae. When these larvae were reared, small nauplii metamorphosed into typical small cypris larvae (240–270 μ) and large nauplii developed into large cypris larvae (280–295 μ). The four other females on the same host emitted numerous nauplii on October 10 and 11. In this case, each female also emitted many small larvae and relatively few large forms. The results of experiments showed that small larvae were destined to become females. Unfortunately, it was not ascertained whether the large larvae were functional male individuals, owing to the small number of the larvae.

(*b*) *Two females on a host-crab* (♂) *of 13 mm. carapace length.* There were 7 females on this host-crab. They measured 7 to 8 mm. in length. Five of them produced exclusively large nauplii which developed into large cypris larvae. The other two females produced both large and small nauplii which developed into large and small cypris larvae, respectively. In this case, the larger larvae were more numerous than the smaller ones.

The chromosomal constitution of the exceptional females described above was examined. It was found that the females which produced many small and few large larvae contained in their ovary oöcytes with 15 bivalent chromosomes and a univalent chromosome (the first maturation division metaphase was examined), while the females that produced many large and few small larvae had oöcytes with 15 bivalent chromosomes. Thus, the exceptional females in question were typical female-producing and male-producing females, so far as their chromosomal constitutions are concerned. Why did these females produce both large and small larvae? Although some tentative explanations might be presented here, the author would like to dismiss them until incontestable evidence is obtained.

Remarks

According to Veillet (1943, 1945), the cypris larvae of *Triangulus galatheae* fall into two groups, the large cypris and the small cypris larvae. Although lacking direct evidence, Veillet maintained that a large cypris develops into an adult

Figures 14–16. Chromosomes of the first maturation division anaphase of the small female-producing egg. A lengthened univalent chromosome is in the middle of the anaphase spindle.

Figures 17–22. Late anaphase of the first maturation division of the small female-producing egg. The univalent chromosome passes to polar body (17–19) or to egg nucleus (20–22).

hermaphrodite, while the smaller one functions as a larval male. In *Sacculina carcini*, according to Veillet, there is no larval dimorphism, but he conjectured that there might be two physiologically different kinds of cypris larvae, one a prospective adult hermaphrodite and the other the larval male. In *Peltogasterella gracilis*, as has been elucidated by the present study, there occurs a marked larval dimorphism. In this species, the large cypris represents the male sex and the small one represents the female sex. A notable fact is that an adult female rarely produces both male and female larvae. It produces regularly either male larvae or female larvae. In *Peltogasterella sulcata* (syn. *Gemmosaccus sulcatus; cf.* Reischman, 1959), Veillet (1952) observed that the cypris larvae derived from adult parasites on a host-crab (*Pagurus cuanensis*) successfully fixed to the crab, while those which were derived from the parasites on two other host-crabs (*P. cuanensis*) failed to fix upon the crab. Veillet made no mention of the reason for such facts. The present author surmises that the cypris larvae derived from the parasites on the one host-crab were female larvae and those from the parasites on the other two crabs were male larvae. The male larvae do not fix to the host-crab.

As examples of animals which are known to have the male-producing and the female-producing females in their population, the aphids (*Aphis* and *Phylloxera*) and the gall-fly (*Neuroteus*) may be mentioned (Morgan, 1912, 1915; Doncaster, 1916). In *Phylloxera caryaecaulis*, Morgan (1912, 1915) found that the eggs which are laid by the female-producing females, *viz.*, the female-producing eggs, are larger than those which are laid by the male-producing female, *viz.*, the male-producing eggs. Morgan observed that during the maturation division of the small male-producing egg two X-chromosomes lag behind the others in the spindle during anaphase, fail to enter the egg nucleus, and pass to the polar body. In the large female-producing egg no elimination of the X-chromosomes occurs. The fate of the egg (whether it develops into a female or into a male) is determined long before maturation division occurs. Why the oöcytes with the same chromosomal constitution develop into two different types of eggs has not yet been settled, although a hypothesis was formulated by Morgan (1915).

As another example of the animal which lays two morphologically and sexually different kinds of eggs, mention may be made of *Dinophilus apatris* (Archiannelida). Here, a female produces both large and small eggs which develop into females and males, respectively. The eggs are laid in a cocoon. The ratio of the large female-producing eggs to the small male-producing eggs is not always 1 : 1, but it varies with females and under influence of external conditions (Korschelt, 1882; Nachtsheim, 1919; Tzonis, 1938). The reason why the same female produces two different kinds of eggs is not yet fully understood. No cytologically distinguishable sex-chromosome has been demonstrated. In *Peltogasterella gracilis* there exists a special chromosome which behaves like a sex-chromosome. The female-producing female possesses this chromosome, while the male-producing female does not. The exact nature and function of this chromosome is unknown, and must be the subject of future research.

The author wishes to express his sincere gratitude to Prof. Atsuhiko Ichikawa (Hokkaido University) for his guidance and criticism during the course of this study. The author also wishes to thank Prof. Tohru Uchida and Prof. Sajiro Makino (Hokkaido University) for their interest and encouragement. Thanks

are due to Prof. Emil Witschi (State University of Iowa) for his kind suggestions. To Dr. Placidus G. Reischman (St. Martin's College) the author is deeply indebted for reading the original manuscript.

Summary

1. The adult females of the rhizocephalan, *Peltogasterella gracilis* (Boschma), although they appear similar, are in reality two kinds, large-egg-producing and small-egg-producing females.

2. The large eggs develop into large larvae which function as males (larval cypris males). The small eggs develop into small larvae which give rise to adult females.

3. In the metaphase plate of the first maturation division of the large male-producing egg 15 bivalent chromosomes are seen, while in the small female-producing egg a univalent chromosome is found beside 15 bivalent chromosomes.

LITERATURE CITED

Delage, Y., 1884. Evolution de la Sacculine. *Arch. Zool. Exp. Gen.*, (II), **2**: 417–736.

Doncaster, L., 1916. Gametogenesis and sex-determination of gall fly, *Neuroteus lenticularis* (*Spathegaster baccarum*). III. *Proc. Roy. Soc. Biol.*, **89**: 183–200.

Ichikawa, A., and R. Yanagimachi, 1958. Studies on the sexual organization of the Rhizocephala. I. The nature of the "testes" of *Peltogasterella socialis* Krüger. *Annot. Zool. Japon.*, **31**: 82–96.

Ichikawa, A., and R. Yanagimachi, 1960. Studies on the sexual organization of the Rhizocephala. II. The reproductive function of the larval (cypris) males of *Peltogaster* and *Sacculina*. *Annot. Zool. Japon.*, **33**: 42–56.

Korschelt, E., 1882. Ueber Bau und Entwicklung des *Dinophilus apatris*. *Zeitschr. wiss. Zool.*, **37**: 315–353.

LaCour, L., 1941. Aceto-orcein. *Stain Technol.*, **16**: 169–174.

Morgan, T. H., 1912. The elimination of the sex-chromosome from the male-producing eggs of phylloxerans. *J. Exp. Zool.*, **12**: 479–498.

Morgan, T. H., 1915. The predetermination of sex in phylloxerans and aphids. *J. Exp. Zool.*, **19**: 285–322.

Nachtsheim, M., 1919. Zytologische und experimentelle Untersuchungen über die Geschlechtsbestimmung bei *Dinophilus apatris* Korsch. *Arch. mikr. Anat.*, **93** (II): 17–140.

Reinhard, E. G., 1942. The reproductive role of the complemental males of *Peltogaster*. *J. Morph.*, **70**: 389–402.

Reischman, P. G., 1959. Rhizocephala of the genus *Peltogasterella* from the coast of the State of Washington to the Bering Sea. *Proc. Kon. Akad. Wetensch. Amsterdam*, (C), **62**: 409–435.

Shirase, S., and R. Yanagimachi, 1957. The early development of *Peltogasterella socialis* Krüger (a rhizocephalan). *Zool. Mag. Tokyo*, **66**: 253–257.

Smith, G., 1906. Rhizocephala. *Fauna Flora Golf. Neapel*, **29**: 1–123.

Tzonis, K., 1938. Beeinflussung der Geschlechtsverhältnisse bei *Dinophilus apatris* Korsch. durch Aussenbedingungen. *Zool. Jahrb. (Physiol.)*, **58**: 539–550.

Veillet, A., 1943. Note sur le dimorphisme des larves de *Lernaeodiscus galatheae* Norman et Scott et sur la nature des "mâles larvaries" des Rhizocephales. *Bull. Inst. Oceanogr. Monaco*, **841**: 1–4.

Veillet, A., 1945. Recherches sur le parasitisme des Crabes et des Galathées par les Rhizocephales et les Epicarides. *Ann. Inst. Oceanogr. Paris*, **22**: 193–341.

Veillet, A., 1952. Metamorphose de la larve cypris du Rhizocephale *Gemmosaccus sulcatus* Lilljborg. *C. R. Acad. Sci. Paris*, **234**: 1310–1312.

Yanagimachi, R., 1961. The use of cetyl alcohol in the rearing of the rhizocephalan larvae. *Crustaceana*, **2**: 37–39.